Identification of Markers for Human Endometrial Carcinoma Stem Cells

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Abstract

Cancer stem cells (CSCs) have recently been identified in many human solid cancers. It was hypothesised that CSCs are responsible for epithelial neoplasia associated with endometrial carcinoma (EC), the most common gynaecological malignancy in women. The aim of this study was to demonstrate that a rare population of EC cells possess CSC properties, and to commence identifying markers for these cells.

Firstly, evidence for a subset of cells with CSC properties in human EC was required. Functional assays revealed that a small population (0.24%) of freshly isolated EC and endometrial hyperplasia cells exhibited the in vitro CSC characteristics of clonogenicity and self renewal. Further assays indicated that rare cells within EC were able to initiate tumorigenic growth, recapitulating the original tumour phenotype when transplanted subrenally in NOD/SCID mice. Importantly, this project discovered that not all cells within EC possessed the same clonogenic, tumour initiating, and self renewal properties, conforming to the CSC hypothesis in all grades of Type I EC, Type II EC and its precursor lesion, endometrial hyperplasia. However, these studies were retrospective and a cell surface marker or combination of markers for the isolation of endometrial cancer stem cells (ECSCs) is required so that they can be isolated, characterised and their role in the development of the disease studied.

To identify potential ECSC markers, advantage was taken of the heterogeneity of EC cells. The hypothesis was that ECSCs would express different markers from their progenitor and more differentiated daughter cancer cells. A panel of 26 potential adult stem cell antibody supernatants was investigated. Since none of the antibodies showed a pattern of reactivity suggestive of CSCs i.e. strong reaction with a small number of EC epithelial cells, a strategy was devised to rank the antibodies into a priority list. This priority list was based on the percentage of cells expressing the marker, the marker’s robustness, and consistency of expression between samples. Based on this priority list, 2 antibodies were tested using functional assays to indicate if they selected for ECSCs.

The CD133-1 antibody (W6B3) was identified as the top potential ECSC marker. Interestingly, the number 3 ranked antibody was the CD133-2 antibody (293-C3). Reports were appearing that indicated there were differences in the expression of these two CD133 epitopes, and thus both were concurrently investigated to determine if either were potential
ECSC markers. Neither antibody enriched for epithelial EC cells with \textit{in vitro} CSC properties. Similar percentages of clonogenic cells (0-0.5%) were observed in the CD133$^+$ and CD133$^-$ (CD133-1 or CD133-2) sorted populations, indicating no enrichment of colony forming units (CFUs). Further, CFUs from both CD133$^+$ and CD133$^-$ subpopulations were equally able to undergo self renewing divisions \textit{in vitro}, with no observable difference between CD133-1 and CD133-2. This data indicated that CD133 did not enrich for ECSCs.

This study lays the groundwork for future studies to explore further markers from the priority list that will enable the prospective isolation of ECSC required to confirm their existence and role in the development of human EC. Together with the identification of normal human endometrial epithelial stem cell markers, the development and progression of EC can be investigated, allowing the identification of potential drug targets selective for CSC, but sparing normal endometrial stem cells. Such treatments will be particularly useful for early stage EC and hyperplasia candidates where the uterus may be conserved, and for late stage cases where hysterectomy is not curative and current treatments target the bulk tumor cells rather than CSCs.
General Declaration

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original papers published in peer reviewed journals and two unpublished manuscripts in preparation for eventual publication. The core theme of the thesis is the identification of human endometrial carcinoma stem cell surface markers. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Centre for Women’s Health, Monash Institute of Medical Research under the supervision of Dr Caroline Gargett.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2 my contribution to the work involved the following:

<table>
<thead>
<tr>
<th>Thesis chapter</th>
<th>Publication title</th>
<th>Publication status</th>
<th>Nature and extent of candidate’s contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Evidence for Cancer Stem Cells in Human Endometrial Carcinoma</td>
<td>Published</td>
<td>I was involved in designing and performing the experiments, collecting and analysing the data for 10 of the 12 figures and tables. I wrote the manuscript except for the sections on serial transplantation methods and results, and the pathological diagnosis of the transplanted tumours.</td>
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</table>

Signed: ........................................................................................................

Date: .............................................
Structure of Thesis

In compliance with Monash University Doctorate Regulation, this thesis consists of published and unpublished works relating to “Identification of human endometrial carcinoma stem cell surface markers”.

Chapter 1 – Introduction. Written as a chapter.

Chapter 2 – Evidence for Cancer Stem Cells in Human Endometrial Carcinoma. Presented as a published manuscript in Cancer Research

Chapter 3 - Identification of Potential Cell Surface Markers of Cancer Stem Cells in Human Endometrial Carcinoma. Written as a manuscript in preparation for publication.

Chapter 4 - CD133-1 and CD133-2 do not Enrich for Endometrial Cancer Stem Cells. Written as a manuscript in preparation for eventual submission.

Chapter 5 – Discussion. Written as a chapter.
List of Publications

Peer Reviewed Publications Relating to the Thesis


In preparation:
S Hubbard, C Gargett. CD133-1 and CD133-2 do not enrich for Human Endometrial Carcinoma Stem Cells.

Book Chapter


Conference Abstracts (presenting author first)

International:


National:

S. Hubbard, R. Chan, C. Gargett. Preliminary evidence for stem cells in human endometrial adenocarcinoma, Southern Health’s Inaugural Research Week, Melbourne, Australia, April, 2007. 1st Prize.


Platform Presentation (presenting author first)

Other Peer Reviewed Publications During PhD

Other Abstracts During PhD
C. Mitchell, S. Hubbard, J. Hirst, W. Giles, T. Zakar. Chronic Suppression of Prostaglandin Endoperoxide H Synthase (PGHS-2) expression in the human amnion by
glucocorticoids in vivo. 51st annual scientific meeting for the Endocrine Society of Australia, Melbourne, August 2008.

Carolyn Mitchell, Thomas Richards, S Hubbard, T Welsh, Pawel K. Zaraycki and Tamas Zakar. Solid phase extraction of prostaglandins E$_2$, F$_{2\alpha}$ and their metabolites from biological samples. 47th annual scientific meeting for The Endocrine Society of Australia, Sydney, August, 2004.


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My supervisor, Dr Caroline Gargett, you have guided me through my PhD, taught me to write and communicate with other scientists. You have critiqued my manuscripts and (many) abstracts. Thank you for providing the opportunities that have helped shape me into a good scientist, your patience and enthusiasm.

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I warmly thank Kjiana Seward and Rachel Chan who helped teach me tissue culture procedures, animal surgery, flow cytometry, and immunohistochemistry, as well as providing much support during the beginning of my candidature. My sincere thanks to Charmaine Tan, Hong Nyugen, and Louie Yi for listening to me during the latter half of my candidature, and good luck with your theses.

For moral support I would like to thank Steven Jackson, my parents and sisters, Rachel and Lydia, and last, but certainly not least, my partner Owen Mattern. Thank you for encouraging me to continue, your words (and drinking nights) kept me going through the many times I wanted to quit.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>micro litre</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABCG2</td>
<td>ATP-Binding Cassette sub-family G member 2</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde Dehydrogenase</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>ASC</td>
<td>Adult Stem Cell</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-Associated X Protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>eDNA</td>
<td>complimentary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CD</td>
<td>Cell Differentiation</td>
</tr>
<tr>
<td>CE</td>
<td>Cloning Efficiency</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<tr>
<td>CK</td>
<td>Cytokeratin</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
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<tr>
<td>DAB</td>
<td>3,3-diamino-benzidine tetrachloride</td>
</tr>
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<td>Abbreviation</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
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<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's Phosphate Buffered Saline</td>
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<tr>
<td>EC</td>
<td>Endometrial Cancer</td>
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<tr>
<td>ECSC</td>
<td>Endometrial Cancer Stem Cell</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>ERα</td>
<td>Oestrogen Receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Oestrogen Receptor beta</td>
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<tr>
<td>ERC</td>
<td>Endometrial Regenerative Cells</td>
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<tr>
<td>ES</td>
<td>Embryonic Stem</td>
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<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorting</td>
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<tr>
<td>FC</td>
<td>Flow Cytometry</td>
</tr>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<tr>
<td>FIGO</td>
<td>The International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorscein isothiocyanate</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid</td>
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<td>hES</td>
<td>human Embryonic Stem</td>
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<td>HJB</td>
<td>Hans-Jörg Bühring</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary Non-Polypoid Colorectal Cancer</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase-conjugated streptavidin</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic Stem Cell</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>LRC</td>
<td>Label Retaining Cell</td>
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<tr>
<td>LSAB</td>
<td>Labelled streptavidin-biotin</td>
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<td>M</td>
<td>Molar</td>
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<tr>
<td>MACS</td>
<td>Magnetic Activated Cell Sorting</td>
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<tr>
<td>Mins</td>
<td>minutes</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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<td>millimetre</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMMT</td>
<td>Mixed Müllerian Malignant Tumour</td>
</tr>
<tr>
<td>MP</td>
<td>Main Population</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite Instability</td>
</tr>
<tr>
<td>n</td>
<td>number of samples</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn Calf Serum</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-Obese Diabetic</td>
</tr>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PBA</td>
<td>Protein Blocking Agent</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF-Rβ</td>
<td>Platelet-Derived Growth Factor-Receptor beta</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
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<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells (erythrocytes)</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
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<td>RNase</td>
<td>Ribonuclease</td>
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<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SC</td>
<td>Stem Cell</td>
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<tr>
<td>SCID</td>
<td>Severe Compromised Immuno- Deficient</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SP</td>
<td>Side Population</td>
</tr>
<tr>
<td>TA</td>
<td>Transit Amplifying</td>
</tr>
<tr>
<td>TIC</td>
<td>Tumour Initiating Cell</td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
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<td>vs.</td>
<td>versus</td>
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Chapter 1: Introduction

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<td>1.7 Aims</td>
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</table>
1.1 Stem cells

1.1.1 What is a Stem Cell?

Currently stem cells (SCs) are defined by their functional properties. Their two defining properties are self renewal and differentiation [1-3]. SCs have several differentiation potentialities. Totipotent cells, e.g. zygotes, have unlimited differentiation potential and can give rise to all cells within an organism [1-4]. Pluripotent SCs, e.g. embryonic stem cells, have a slightly more restricted potential, differentiating into all cell types of the three germ layers (mesoderm, endoderm, and ectoderm) and germ cells [1-4]. Multipotent SCs refer to adult stem cells (ASCs) which are present from the foetal stage onwards. These cells have a more restricted differentiation potential, only giving rise to cells of the organ in which they reside. However, this concept of restricted differentiation potential is currently being challenged [1-4].

1.1.2 Embryonic Stem Cells

Embryonic stem (ES) cells are derived from cells from the inner cell mass of the blastocyst or from primordial germ cells [1, 4-6]. ES cells have been successfully cultured from mouse blastocysts since 1981 and more recently (late 1990’s) from human blastocysts and germ cells [1, 5-8]. Their pluripotency has been demonstrated by their ability to contribute to all tissues of a chimeric offspring when re-injected into a host’s blastocyst and they formed teratomas when injected directly into nude mice, a defining feature of ES cells [1, 3, 5, 6, 8]. Also, hES (human ES) cells formed embryoid bodies which spontaneously differentiated into a complex of tissues comprising, cardiomyocytes, endothelial cells, fibroblast-like cells, spontaneously contracting smooth muscle-like cells, striated muscle cells, erythroid cells, and adipocytes [1, 5, 9-13]. This potential to differentiate into multiple cell lineages makes ES cells a promising source of cells for regenerative medicine purposes. Many medical researchers hope to direct the differentiation of hES cells into particular cell types, by culturing them with specific growth factors or other inducing agents, in order to cure diseased or injured tissues.

hES cells have been differentiated into many specific cell lineages in vitro, with concomitant expression of cell-specific protein and gene markers, and in some cases performing the functions associated with the differentiated cell types. Researchers have
been able to differentiate hES cells into adipocytes [9], smooth muscle cells [10], vascular cells [14], multiple haematopoietic cells [13, 15], mesenchymal stromal cells [16], tendons [17], hepatic-like cells [18], various neural cells [1, 19-22] and epithelial cells [23]. hES cell derived neuronal precursor cells produced myelin sheaths in 6/9 rat transplants when implanted into myelin deficient rats [20]. However, this study failed to demonstrate any change in the physiological status of the rats, but encouragingly the implanted cells did not revert to an undifferentiated state post implantation or produce teratomas. Several subsequent studies have demonstrated the reversal of disease or injury effects with the transplantation of hES cell derivatives in animal models, including spinal cord defects and motor neuron injury [24-27], Parkinson’s disease [28] and visual disease [29]. In early 2009 Geron received FDA clearance to begin human clinical trials with derivatives of hES cells to treat subacute thoracic spinal cord injuries [30]. However, this approval is currently under review as some animals developed cysts. Interestingly, a recent publication using hES cell derived oligodendrocyte progenitor cells to treat cervical injuries demonstrated an improvement in forelimb function [31].

1.1.3 Adult Stem Cells

Unlike ES cells, ASCs are rare cells isolated from the organ in which they reside, where they function to replace cells lost to natural attrition and injury (Fig 1.1) [6, 32]. ASCs and adult progenitor cells have been identified in a number of tissues including the haematopoietic system, neural system, intestines, testis, breast, uterus and the ovaries [32-35].

1.1.3.1 Proliferation of Adult Stem Cells

ASCs are relatively quiescent (Fig 1.1) [1, 6], proliferating in response to environmental signals [1]. In some studies putative ASCs have been demonstrated to proliferate quickly [36], while others have demonstrated that they proliferate slower than other adult cells. In vitro ASCs proliferate to form clones of cells [35]. This property is examined by seeding freshly dissociated single cells at low cell densities in adherent culture [35, 37, 38]. Individual cells attach to the plates and some give rise to clones of cells, which can be stained and counted to determine the cloning efficiency (CE). The CE of epithelial cells from various human tissues ranges from 0.9%-12.3% [39, 40]. However, the CE may be an overestimation of the number of ASCs in a tissue as more differentiated cells (i.e. transit
amplifying [TA] cells) are also able to initiate clones. However, progenitor and TA derived clones often have different growth rates and clonal characteristics compared to ASC derived clones [37]. Further, the culture environment may not permit the full differentiation of ASCs or support the proliferation of all cell types. Culture also often induces premature terminal differentiation of the ASC. However, this assay is quantitative, easy to perform, and useful as a screening assay to detect ASC activity in isolated tissues [35].

![Figure 1.1 Adult Stem Cell Hierarchy.](image)

The adult stem cell (ASC) has the largest capacity for self renewal and is able to produce more differentiated daughter cells, while remaining relatively quiescent. Progenitor cells are more differentiated than the ASC and retain limited self renewal capacity. Transit amplifying (TA) cells proliferate rapidly, producing the differentiated daughter cells of the organism. From Chan et al. (2004) [101].

The ability of ASCs to proliferate and form colonies has also been studied in vivo. Mouse haematopoietic colony forming units (CFUs) were identified by Till and McCulloch (1961) when they injected irradiated mice with nucleated bone marrow cells and observed the formation of colonies in the spleen [41-44]. Further investigations indicated that these colonies were initiated by a single cell [2, 42] and that the rapidly appearing colonies were initiated by progenitor cells, while the slower cycling haematopoietic stem cells (HSCs) initiated colonies more slowly, appearing at later time points [43, 44].
1.1.3.2 Self Renewal of Adult Stem Cells

In contrast to hES cells, human ASCs have limited self renewal activity in vitro and in vivo. The purpose of self renewal of ASCs is to maintain a small population of ASCs with differentiation capacity throughout the lifetime of the organism (Fig 1.1). Studies on non-immortalised, diploid, human cells demonstrate that they reach senescence between 40-150 passages [6, 32, 45-47]. This indicates that ASCs are unable to continually self renew and exhaust their proliferation potential in 2D culture conditions. Further, clones potentially generated by ASCs can be dissociated into single cells and replated, with some single cells regenerating clones with the same morphology as the parent clone. This demonstrates self renewal in vitro, however, this re-cloning potential is limited [48, 49]. A similar phenomenon is observed in putative human ovarian SC cultures, where the potential ovarian ASC proliferates in culture producing daughter cells that are morphologically identical to the parent cell [36]. Additionally, haematopoietic colonies generated in mouse hosts and re-injected into another mouse formed new colonies with a similar differentiation pattern to that observed in the original host, demonstrating the self renewal of ASCs in vivo [2, 50, 51].

1.1.3.3 Differentiation of Adult Stem Cells

ASCs appear to retain the capacity to differentiate in culture [52] and in vivo. ASCs need to generate differentiated progeny of the organ in which they reside to maintain homeostasis (Fig 1.1). Differentiation in vitro is determined by the expression, or lack thereof, of tissue specific markers [38, 53]. In vitro, HSCs are able to give rise to clones containing cells expressing differentiation markers associated with different blood lineages. Putative ovarian SCs give rise to granulosa and oocyte-like cells after 3 days of culture [53-55]. Differentiation of potential epithelial prostate and mammary SCs cultured in matrigel form clumps of cells with side-branches resembling ducts, and are composed of cells expressing unique sets of tissue specific differentiation markers [35, 37]. Also, some clones and cultures demonstrate self renewal and differentiation by producing identical cells and clones and/or cultures of mixed or entirely different morphologies [38, 40, 48].

Transplantation of human cells into animal hosts demonstrates the ability of ASCs to differentiate in vivo when the parent tissue is recapitulated in the newly formed tissue. Transplanted single cell suspensions of human mammary epithelial cells combined with
stromal cells gave rise to histologically normal human mammary epithelial outgrowths, that proliferated, expressed the two epithelial lineage markers (luminal and myoepithelial), and appeared to respond appropriately to pregnancy hormones [35, 56]. While reconstitution of the parent tissue *in vivo* is the gold standard for evidence of ASC activity, this approach is expensive, time consuming, and not amenable for high throughput screening [35]. Further, transplantation assays do not distinguish between ASCs and adult progenitor cells, which may also give rise to growths, though these are thought to be smaller with less differentiation [35].

**1.1.3.4 Adult Stem Cells and the Treatment of Disease**

Similar to hES cells, human ASCs may have potential to treat diseases and injury. HSCs have been used in unpurified form to treat human leukaemias for over 40 years [57-59]. They have also been used to treat lymphoma [60], severe aplastic anaemia [61, 62], and other blood disorders [63, 64]. More recently, other ASCs have been used to treat cardiac injury [65-67], patella injury [68] and thoracic diseases [69, 70]. Treatment of patella injury and thoracic diseases shows promise, although these initial studies were performed on a small number of patients (≤ 5) and under non-randomised conditions. Substantial progress has been made on the use of ASCs for treatment of cardiac injury, with two separate randomised studies, of approximately 60 patients, each reporting improvement in cardiac function after at least six months following injection of progenitor cells [66, 67].
1.2 Adult Stem cells in the Normal Endometrium

1.2.1 The Structure of the Endometrium

The uterus is a pear shaped organ in the female pelvic cavity [71, 72]. In nulliparous women it measures 8 cm long, 5 cm wide and 2.5 cm thick [71]. The uterine body, or uterine corpus, tapers to a narrow neck termed the cervix, which in turn opens into the vagina [73]. The uterine mucosa, the endometrium, lines the cavity of the uterus and is where fertilised ovum implant (Fig 1.2) [71-73]. The surrounding smooth muscle is called the myometrium and contracts during parturition [71-74].

The endometrium is composed of psuedostratified columnar, ciliated epithelium that forms simple tubular glands, which are supported by a cellular stroma [72, 74, 75]. There are three histologically distinct layers in the endometrium [72]. The deepest layer is the stratum basalis, adjacent to the myometrium, which remains during and after menstruation, and is composed of the lower section of the glands and dense stroma (Fig 1.2) [72, 74, 76]. The intermediate layer (stratum spongiosum) and the thin superficial layer (stratum compactum), comprising the top section of the glands, are shed during menstruation and undergo significant changes during the cycle [72, 76]. Collectively, the intermediate and

**Figure 1.2 The Histology of the Endometrium.** The basalis sits on top of the myometrium and remains during menstruation. Above this layer is the functionalis which regrows each month after being shed during menstruation. From Gargett et al. (2007) [74].
superficial layers are termed the stratum functionalis and regenerate from the basalis following menstruation (Fig 1.2) [76, 77]. Oestrogen and progesterone are the hormones regulating the cyclical changes in the human endometrium functionalis [74, 76, 78].

### 1.2.2 The Menstrual Cycle

The standard human menstrual cycle is 26-32 days and is divided into three phases, the proliferative, secretory, and menstrual phases (Fig 1.3) [77, 78]. These cycles of growth, differentiation, and regression prepare the endometrium for implantation of an embryo. Day 1 of the cycle is the first day of menstrual bleeding and technically the cycle begins with the menstrual phase. However, it is easiest to describe the cycle beginning at the proliferative phase. It is important to note that the morphological changes occur in the functionalis, not the basalis [79].

![Figure 1.3 The Menstrual Cycle](image)

**Figure 1.3 The Menstrual Cycle.** The cycle begins with menstruation, where the functionalis is shed. During the following proliferative phase the functionalis regrows from the basalis. After ovulation the endometrium enters the secretory phase where the glands secrete a milieu for nourishing an embryo. When implantation does not occur, progesterone levels drop, leading to the regression and shedding of the terminally differentiated endometrium functionalis, and the cycle begins again. From Gargett et al. (2008) [34].

The proliferative phase begins around cycle day 5 and under the influence of rising oestrogen levels which dominate this phase (Fig 1.3) [34, 77, 80, 81]. Oestrogen induces cellular proliferation of all the endometrial cellular components [34, 76, 79-86]. During this time narrow, short, straight and partially collapsed glands of the thin endometrium
thicken and elongate to form branched glands, which start to become tortuous by the late proliferative phase [34, 76, 77, 87]. Oestrogen’s actions are mediated through its receptors, oestrogen receptor α (ERα) and ERβ [34, 77, 78, 80, 86, 88]. ERα is the dominant subtype in the functionalis, which is maximally expressed during the proliferative phase and declines in the secretory phase, as does the proliferative action of oestrogen [34, 77, 80, 86, 88]. ERβ is also expressed in the epithelium, at much lower levels than ERα, but not in the stroma, yet it is the only ER found in the endothelium [34, 77, 78, 86]. ERβ is also down regulated in the secretory phase [34, 77, 80, 86]. Also, at the beginning of this phase the tight junctions that isolate the glandular lumen from the internal environment start to break down, resulting in an increase in extracellular spaces between the cells [89].

The secretory phase follows ovulation (~cycle day 14) and progesterone induces differentiation of the endometrium in preparation for embryo implantation (Fig 1.3) [76-78, 80]. Progesterone suppresses oestrogen driven mitosis in the glands and stroma by downregulating ERα expression [77, 80, 86]. The glands become increasingly tortuous and the epithelial cells begin secretory activity, stromal oedema occurs, and the spiral arterioles begin to coil [76, 77, 80]. Progesterone mediates its actions through progesterone receptors (PR) –A and PR-B [77, 78, 80, 90]. The expression of these receptors is stimulated by oestrogen in the proliferative phase and is down regulated by progesterone in the secretory phase [77, 78, 80, 86]. In the glandular epithelium both isoforms are expressed, but only PR-B persists in the mid secretory phase [77, 78, 80, 86]. In the stroma PR-A is the dominant isoform, and is associated with decidualisation [77, 78, 80, 86, 90]. Decidualisation of the stromal cells occurs in the mid secretory phase, around cycle day 22, where they take on an epithelial-like appearance, enlarge, and become secretory [77]. This process also involves remodelling of the vasculature and extracellular matrix, as well as leukocyte infiltration [77]. Decidualisation is required for the endometrium to be receptive for implantation of a blastocyst, and only occurs in the presence of progesterone in an oestrogen primed endometrium [77, 78]. If pregnancy does not occur, progesterone (and oestrogen) levels fall, triggering endometrial regression and menstruation [76-78].

During the menstrual phase, the extracellular matrix degrades and the functionalis layer is shed [76-78]. Scanning electron microscopy studies on human hysterectomy samples indicated that re-epithelialisation precedes stromal expansion and is initiated from remaining basalis glands that are free from overlying degenerated tissue, as well as from
any persisting areas of surface epithelium [77, 78, 80, 91]. The proliferative phase then follows and the cycles continue until pregnancy or menopause.

1.2.3 Evidence for Stem Cells in the Normal Endometrium

1.2.3.1 Indirect Evidence

Over many years the indirect evidence for potential ASCs in the human endometrium has accumulated [74]. Primarily, the human endometrium is a highly regenerative tissue that monthly undergoes major remodelling associated with the proliferation, differentiation and breakdown of the tissue [74]. It is also able to regenerate after extensive curettage, parturition, ablation, and when post menopausal women are given oestrogen replacement therapy, and in rare cases is able to support a pregnancy after ablation and resection [74, 92-94]. These observations suggest that ASCs exist in the human endometrium. Further, individual human and mouse endometrial glands are clonal in origin, as detected by PCR and immunohistochemical methods [95-97], indicating that each gland is generated from a single precursor cell. Some evidence has also suggested that proximally located glands may be generated from the same precursor cell [95]. Also, the counting of epigenetic errors in selected genes has indicated an ASC origin of the endometrial glands [98]. During each cell cycle epigenetic variants arise, and in the cycling endometrium persistent polymorphisms indicate descendants from a persisting cell, an ASC, as epigenetic variation in transient amplifying cells would be lost during menstruation [74, 98]. Epigenetic variations were found to increase with age until menopause, indicating the presence of ASCs that are maintained in the endometrium throughout the life of the woman [74, 98]. Different methylation patterns within the same uterus were considered indicative of individual glands being maintained by distinct SC niches, containing up to 64 SCs, that evolve independently [74, 98].

It has been hypothesised that the human endometrial ASC resides in the basalis, from which the functionalis derives, evidenced by the re-epithelialisation from the basalis stumps (Section 1.2.2). This indicates the likelihood that a highly proliferative epithelial stem/progenitor cell is present in the endometrial basalis. Studies in menstruating primates have found that epithelial cells in the basalis zone are not mitotically inhibited by progesterone during the secretory phase and continue to proliferate producing cells that may form part of the functionalis soon after menstruation [74, 84, 99, 100]. Other evidence
has suggested that the endometrial ASC may be a blood derived cell [96]. Females with HLA mismatched donor bone marrow transplants possessed focal areas of donor derived cells in their endometria, in both the stromal and epithelial compartments [96]. However, it remains to be determined if this occurs in normal healthy women whom have not undergone bone marrow transplants. Also, it is not known if these chimeric endometria comprising a significant proportion of bone marrow derived cells could support a pregnancy.

1.2.3.2 Direct Evidence

1.2.3.2.1 In vitro Evidence

Rare cells within the human endometrium have been demonstrated to possess the characteristics of ASCs. In vitro studies have demonstrated that 0.22% and 1.25% of cells from the epithelial and stromal compartments, respectively, are able to form large clones of cells from samples taken at any stage of the menstrual cycle, although there was noteworthy variation between samples [74, 101, 102]. A similar number of cells were able to form large clones when investigated by the limiting dilution technique [39]. The clonogenic cells underwent self renewal and had a high proliferative potential, two properties of ASCs [39, 74, 101]. A higher percentage of cells were able to initiate small clones, but further analysis (lower proliferative potential when compared to the cells instigating the large clones and inability to undergo self renewing divisions) lead to the conclusion that these were initiated by TA cells [39, 74, 101, 102]. Clonogenic cells initiating large epithelial clones also lacked epithelial markers (cytokeratin, epithelial cell adhesion molecule), but expressed α6 integrin, indicating their undifferentiated state, whereas the smaller epithelial clones expressed differentiation markers when examined by immunohistochemistry [101]. The large epithelial clones were also able to differentiate into large, cytokeratin positive, gland like structures in matrigel, another SC trait [39]. Large stromal clones expressed CD29, CD44, CD73, and CD90, with some expressing CD105 and CD146 [39], typical surface markers of bone marrow and adipose tissue mesenchymal stem/stromal cells. These are potential markers for ASCs and indicate that some large stromal clones may be initiated by endometrial stromal stem/progenitor cells. Gene expression profiling of the large vs. small clones may provide insight into the different properties of the cells initiating the clones [74].
Clonogenic cells have also been identified in inactive, atrophic human endometrium [102]. The percentage of cells able to form large clones was not significantly different from that found in normal cycling endometrium [102]. This suggests that functional stem-like cells remain in atrophic human endometrium after menopause, and that these cells can be reactivated under certain hormonal conditions or perhaps inappropriately in disease causing conditions [102].

1.2.3.2.2 In vivo Evidence

Single isolated human endometrial cells (5 x 10^5) transplanted under the kidney capsule of ovariectomised NOG (NOD/Shi-scid/IL-2Rγnull) mice reconstituted endometrial–like structures after hormone treatment [103]. The stroma-like tissue stained positive for the normal human endometrial stromal markers, CD10, vimentin and CD13; and the pseudostratified gland-like structures were positive for normal human endometrial gland markers, including cytokeratin and CD9 [103]. When the mice were treated cyclically with oestrogen and progesterone the stromal cells reacted normally, producing prolactin, and the glands became tortuous. Progesterone withdrawal resulted in glandular cysts and haemorrhage [103], indicating that cells within the normal human endometrium are able to produce endometrial structures in vivo, a property of ASCs. However, limiting dilution investigations are still required to determine the minimal number of cells for endometrial tissue reconstitution, and if the stromal, glandular, and even myometrial cells give rise to all the structures of the uterus or just the tissue types from which they originate.

Label retention studies in the mouse endometrium have indicated that ASCs reside here [104]. While the mouse does not undergo menstruation, the murine endometrium does undergo a four day cycle of proliferation and apoptosis, and also undergoes substantial regeneration on administration of oestrogen in ovariectomised mice, again indicative of ASCs [74]. Label retention studies rely on the differential dilution of DNA synthesis labels, e.g. BrdU (Bromodeoxyuridine), in ASCs vs. TA cells [105]. BrdU is a thymine analogue [104-106], which is incorporated into genomic DNA during the replicative phase of the mitotic cycle and is progressively diluted with each cell division [104-106]. The label retaining cell (LRC) is a relatively quiescent cell, a property of ASCs [104]. Around 3% of epithelial and 6% of stromal cells were identified as LRCs in the mouse endometrium [74, 104]. In general LRCs were present as single cells in the luminal epithelium or stromal cells adjacent to luminal and glandular epithelium or near the
endometrial-myometrial junction [74, 104, 106]. The LRCs did not co-localise with CD45 or CD31 indicating that they were not bone marrow derived or endothelial cells [74, 104]. Some stromal LRCs were perivascular and co-expressed α-smooth muscle actin [74, 104]. Another study also found stromal LRCs, at much lower numbers, in the endometrial-myometrial junction [106]. A small percentage of these appeared to express c-kit (CD117) and Oct-4, haematopoietic pluripotency and self renewal markers [74, 105, 106]. ERα was present in approximately 16% of stromal LRCs, often in cells with close proximity to the luminal epithelium. However, epithelial LRCs themselves did not express ERα, but were adjacent to cells expressing ERα [74, 104]. Epithelial LRCs proliferated (Ki-67+) after oestrogen stimulation, indicating that they do not receive direct signals via ERα, but rather respond to paracrine signals from adjacent niche cells [74, 104, 105]. This finding may be particularly significant if human endometrial ASCs are identified.

1.2.3.2.3 Expression of Potential Stem Cell Markers in the Human Endometrium

PCR has revealed that a number of human endometrial samples express a variety of self renewal and pluripotency genes, potential genetic markers of ASCs [107-110]. However, only a few of these genes or their products have been investigated using immunohistochemical methods. Both methods have produced conflicting results. SALL4 was found in the human endometrium using RT-PCR, but it was absent from immunohistochemically stained sections [107]. This contradictory result for SALL4 may be explained by the rareness of SCs, which may not have been present in the few sections studied. Perhaps if more sections were examined positive cells would be observed. For the pluripotency marker OCT4, conflicting results have also been obtained. One study failed to detect OCT4 by RT-PCR [107], whereas another detected OCT4 in human endometrial samples [109], although both found single OCT4 stained cells by immunohistochemistry [107, 109]. There are many pseudogenes for OCT4, and it is possible that the PCR primers were specific for one pseudogene and the antibody recognised a number of OCT4-like proteins. Unfortunately, neither study separated epithelial and stromal cells and thus did not identify which cells expressed OCT4 [74]. Further work is required to determine if either cellular compartment has a stronger expression for the SC related genes and also if OCT4 expressing cells are located in the basalis or functionalis layers.
Other ASC developmental pathways have been investigated in the human endometrium. The intestinal ASC marker, \textit{MUSASHI-1}, was localised to single cells or small groups of stroma cells within the vicinity of glands, or in epithelial cells [108]. Interestingly, this is a similar location to some of the LRCs in the mouse endometrium [104]. The \textit{MUSASHI-1} positive cells co-localised with \textit{NOTCH-1} and expressed telomerase, which are other potential SC related genes, indicating that \textit{MUSASHI-1} may mark ASCs within the human endometrium [108]. Further, there was stronger expression in the basalis when compared to the functionalis, the potential location of the endometrial SCs. The expression was increased in the proliferative phase compared to the secretory phase, a time when the SCs are thought to be actively cycling. Similarly, the expression of the cell surface markers CD117 (c-kit) and CD34 have been investigated in the human endometrium from the foetal stage until after menopause [111], with the hypothesis being that ASC markers should persist from development until death. CD117 was present in both the stroma and glands in the foetal stage, reproductive stage, and in the atrophic endometrium [111]. It was also present in the pregnant uterus [111]. CD34 was also expressed in the foetal, reproductive and atrophic endometrial stroma, adjacent to basalis glands, but was absent from gestational uteri [111]. This may indicate that CD34 marks the stem/progenitor cell that does not activate with pregnancy. It is possible that TA cells, daughter cells of endometrial ASCs, may also express some SC related genes [108].

\textbf{1.2.3.2.4 Side Population Cells as Endometrial Stem/Progenitor Cells}

Side population (SP) cells are cells capable of effluxing the vital dye Hoechst 33342, often due to the expression and function of the transporter ABCG2. When analysed by dual wavelength flow cytometry, a small population of cells are visualised on the side of the main population (MP) of cells, and hence is termed the SP. SP cells were first identified in the haematopoietic system and this technique may partially purify SCs [112]. Small percentages (0.5-1.1\%) of unfractionated human endometrial cells have been identified as SP cells throughout the menstrual cycle [113, 114]. These cells are a heterogeneous population comprising stromal, epithelial and endothelial cells, and express other potential SC surface markers [113]. The SP cells were also able to initiate larger colonies than MP cells in clonal culture, indicating that the endometrial ASCs may be located within this fraction [113]. When the human endometrium was separated into mainly glandular and mainly stromal cells, SP cells were found in both compartments and these cells were not contaminating blood or endothelial cells [114]. Under the correct culture conditions the SP
cells from both the epithelial and stromal compartments formed clones and grew for at least 9 months, whereas the MP cells were senescent at 3 months [114]. This longer proliferation potential and survival are properties of ASCs. Within 5 months, epithelial derived SP clones grew into gland-like structures expressing CD9 and E-cadherin, but not CD13 or vimentin, demonstrating differentiation in vitro [114]. The stromal derived SP clones formed clumps that expressed CD13 and vimentin, but not CD9 or E-cadherin [114], indicating that the SP cells from each compartment may be progenitor cells restricted in producing either epithelial or stromal cells.

1.2.3.2.5 Evidence for Mesenchymal Stem Cells in the Human Endometrium

There is evidence for mesenchymal stem cells (MSCs) in the human endometrium. MSCs are able to differentiate into the four mesenchymal lineages, myogenic, osteogenic, adipogenic, and chondrogenic. In vitro, large human endometrial stromal clones appear to differentiate into these lineages when cultured in specific induction media [39, 102, 115-117]. Interestingly, cells from the myometrium, fallopian tube, and uterosacral ligament were unable to undergo chondrocytic differentiation [117]. Co-expression of CD146 and PDGF-Rβ (platelet derived growth factor-receptor β) was used to partially purify multipotent MSCs from the human endometrium [115]. These stromal cells produced more and larger clones than the CD146-PDGF-Rβ− cells, and these larger clones differentiated into osteogenic, adipogenic, myogenic and chondrogenic lineages based on RNA expression and immunohistochemical analysis [115]. MSCs appear to be shed during menstruation as MSC-like cells have been cultured from menstrual blood [118, 119]. When single cells cultured from the menstrual blood were plated in 96 well plates some cells, termed endometrial regenerative cells (ERCs), formed clones that generated cell lines that expressed a wide variety of potential MSC and other ASC markers [119]. Further, ERCs were differentiated into the MSC lineages [119], including cardiomyocyte like cells, which were transplanted into nude rat’s hearts after induced myocardial infarction [120]. Finally, in humans, 9-30 million donor derived cultured ERCs were injected into multiple sclerosis patients and did not cause adverse reactions [121]. This is a promising start for the treatment of human disease with ERCs, however, patients were receiving other treatments [121] so it is still to be determined if ERCs are able to incorporate into patient tissues and reverse the effects of injury or disease.
1.3 Cancer Stem Cells

1.3.1 The Hallmarks of Cancer

There are over 100 different types of human cancer. Cancerous cells distort the normal regulatory circuits, undergoing uncontrolled proliferation and altered responses to normal homeostatic mechanisms. In 2000, Hanahan and Weinberg [122] suggested a set of six genomic alterations that governed the malignant transformation of normal human cells into cancerous ones [122]. They deduced that since mammalian cells carry similar molecular machinery, that the acquired capabilities of human cancer cells occur through similar molecular pathways [122]. They further theorised that tumourigenesis is a multistep process where the first mutation confers a growth advantage, making these cells more susceptible to further genetic alterations [122]. The six alterations are: sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [122]. These molecular alterations have been widely accepted by many scientists, who now try to prove these mechanisms exist in the development of a particular cancer.

1.3.1.1 Growth Factor Signalling

Normally, growth signals induce cells to proliferate. In cancer, many tumour cells produce their own growth signals, disrupting normal homeostatic mechanisms which normally prevent excessive proliferation, motility and apoptosis. Cancer cells may also over-express growth factor receptors such as HER-2/neu, or these receptors may be abnormal and constitutively active, leading to ligand independent signalling or a hyper-responsiveness to normal growth factor levels [122-124]. Self-sufficiency in growth signals can also be acquired by altering downstream effector molecules, such as Ras, which becomes constitutively active, resulting in ongoing target gene stimulation [122, 123, 125, 126].

1.3.1.2 Failure of Anti-Proliferative Signals

Similar to proliferative signals, antigrowth signals operate within the normal system to maintain homeostasis by blocking proliferative signals and inducing cells into quiescence or a postmitotic state [122, 127, 128]. Many cancers have altered or disabled antiproliferative signals, often via the retinoblastoma protein or its associated proteins, p107 and p130, integrins or other cell adhesion molecules [122, 127-130]. These
alterations include downregulation of antiproliferative receptors, mutations in the receptors rendering them dysfunctional, and overexpression of oncogenes that impair differentiation and promote growth [122, 127, 128, 130].

1.3.1.3 Apoptosis

Many tumour cells evade apoptosis. Apoptosis is a tightly regulated process of cell death, often involving the mitochondria, p53 and Bax proteins, induced by environmental cues [122, 131]. These cues include internal abnormalities such as DNA damage, oncogene hyperexpression, or hypoxia [122, 131]. Mutated p53 has been identified in many cancers, often resulting in this tumour suppressor protein becoming non-functional and hence dysregulation or lack of apoptotic signals following DNA damage. Mutations in the apoptosis sensors or the proteins they affect can prevent the cancerous cell from responding to apoptosis signals that would normally result in its removal [122].

1.3.1.4 Infinite Replicative Potential

The above three acquired characteristics (sections 1.3.1.1-3) are not sufficient to ensure tumour development [122]. Cancerous cells also need to acquire an infinite replicative potential which is not present in normal cells (with the somewhat exception of normal ASCs), or else the cancer cells die [122, 132]. When tumour suppressor genes are disabled normal cells survive longer in culture, but eventually reach crisis where there is massive cell death because of telomere shortening [122, 132]. Telomeres are hexanucleotide sequences located on the end of chromosomes that are depleted by 50-100 base pairs with every cell cycle [122, 133, 134]. If a cell becomes immortalised via the activation of the telomere extension enzyme, hTERT, then it survives through the crisis period and is able to replicate indefinitely [122, 133, 134]. It is assumed that a similar situation occurs in vivo, where tumour suppressor genes become disabled and the cancerous cells are able to activate the telomere extension enzymes. Alternatively, the loss of telomeres from the ends of chromosomes results in “sticky” ends that fuse with other chromosomes producing karotypic disarray and sometimes tumour suppressor genes are lost e.g. p16 [122].

1.3.1.5 Angiogenesis

Organs require vasculature to provide nutrients and remove wastes. Tumours must develop blood and lymph vessels if they are to survive, prosper and generate large sized lesions.
This process is called angiogenesis [122]. This angiogenic response may be accomplished by altering the balance of angiogenic inducers and inhibitors, such as upregulating vascular growth promoters including vascular endothelial growth factor and fibroblast growth factor, or by down regulating vascular growth inhibitors such as thrombospondin-1 and β-interferon [122, 135]. Interestingly, thrombospondin-1 is positively regulated by p53. When p53 is mutated and non-functional there is a consequent down regulation of thrombospondin-1 [122], thus promoting vessel growth into the tumour.

1.3.1.6 Metastasis

Metastasis is the cause of 90% of human cancer related deaths [122]. Tumour tissue invasion and metastasis involves downregulation or changing the expression of certain cell-cell adhesion molecules, including cadherins and integrins, upregulation of proteases that degrade the extracellular matrix, and downregulation of protease inhibitors, facilitating cell motility [122, 136, 137]. Many epithelial cancers downregulate E-cadherin, a cell-cell adhesion molecule, resulting in motile cells which may establish a metastasis if they acquire the other cancer related capabilities discussed above (sections 1.3.1.1-5) [122, 137, 138]. While tethering the cells in place, the cell-cell adhesion molecules also convey regulatory signals to the cells, meaning that the loss of a single protein function can have multiple effects in the progression to cancer and metastasis.

While many signalling pathways require alteration to facilitate cancer development, it is important to note that modifications in some genes result in adjustments in more than one pathway. For example the mutation in the p53 tumour suppressor gene may permit the insensitivity to apoptotic factors as well as inducing angiogenesis, while at the same time allowing cells with damaged DNA to continue cycling, resulting in an increased probability of acquiring further genetic changes.

Hanahan and Weinberg hypothesised that by 2020 the cellular signalling pathways would be completely mapped, allowing investigation of genome wide expression profiles of tumours [122]. This in turn would then allow us to determine if all cancers required all or only some of the six characteristics described above, or if other undiscovered characteristics were also necessary. Unfortunately, these signalling circuits have proved to be highly redundant and genetic alterations including multiple reading frames and epigenetic changes have complicated the picture. While these six hallmarks may be
required for all human cancers, understanding the molecular mechanisms governing them still seems some way off. In order to better understand these molecular mechanisms, cells that maintain and initiate cancer growth need to be investigated to determine the exact genetic and molecular lesions acquired during the tumourigenic process.

1.3.2 The Cancer Stem Cell Concept

Many human cancers are composed of cell populations with heterogeneous immuno- and genetic profiles, proliferation potentials and differentiation capacities [139, 140]. To accommodate this heterogeneity the cancer stem cell (CSC) hypothesis has been proposed. CSCs are defined as a subset of tumour cells with the capacity to self renew and give rise to the differentiated tumour cells that comprise the bulk of the tumour [139, 141, 142]. It also suggests that tumours are similar to an organ where cells exist in a hierarchical arrangement. This theory was devised for leukaemic cells, where the CSC represented < 1% of the total cell population [143, 144]. It is hypothesised that CSCs express cell surface markers or a combination of specific markers that distinguish them from the bulk of the tumour population. Further, these markers can be used to purify populations of CSCs in much the same way that normal SCs can be isolated from the remaining cells in the organ in which they reside. The CSC theory, although controversial, has important implications for our understanding of the biology and development of cancers, and in particular as targets for the development of novel treatments.

Differences in proliferation potentials of tumour cells have been observed in numerous cancers, where it has been demonstrated that only a few cells from the cancer form clones in vitro [142, 145-148]. Also, autotransplantation of malignant cells in humans does not always establish a tumour [139, 149]. Further, not all human cancer cells xenografted into immunocompromised animal models established tumours [143, 148, 150-152], indicating that only certain cells within the tumour retain the capacity to establish, maintain and promote the development of the tumours, supporting the CSC hypothesis.

1.3.2.1 Properties of Cancer Stem Cells

By definition, CSCs have similar properties to ASCs. CSCs may not necessarily acquire the first genetic mutation that initiates tumourigenesis, but they are the cells that maintain the tumour over time [139]. However, subsequent mutations within a cancer may create new CSCs that overtake or coexist with the older CSCs [139]. Whatever the mutation, the
CSC must retain the capacity to self renew. To date, the best *in vivo* indicator of CSC self renewal is the serial transplantation of CSC enriched populations into immunocompromised mice (Fig 1.4), where the tumour is re-established with a similar phenotype to the original tumour in each serial xenografting [139]. This technique also demonstrates that CSCs are able to give rise to large numbers of differentiated progeny. CSCs may also be relatively quiescent, allowing them to escape the cytotoxic effects of chemotherapeutic agents targeting rapidly cycling cells. Chemotherapeutic failure can also be acquired by upregulating drug transporters [139, 141], which rapidly transport harmful agents from the cell before exerting their effects. Emerging evidence also indicates that CSCs may be less radiosensitive than the majority of cancer cells [153], with the hypothesis that CSCs possess DNA protective mechanisms that minimise the damaging effects of radiation [153].

**Figure 1.4 In vitro and In vivo assays for Cancer Stem Cells.** A) Tumour cells may be passaged directly onto tissue culture ware or embedded in matrigel, where they proliferate to form clones and colonies [139]. B) Orthotopic transplantation in immunocompromised mice evaluates for CSCs within a tumour [139]. Adapted from Visvader and Linderman (2008) [139].

Recent evidence indicates that CSCs may not be as rare as originally thought [139, 141]. In normal organs, homeostatic mechanisms tightly regulate the proliferation of the normal ASCs, maintaining their numbers at relatively low levels. In cancer these mechanisms no longer apply and CSCs may self replicate to expand the mutated SC pool [141]. However, numbers of CSCs are often inferred from the percentage of cells expressing a particular surface marker phenotype which may also be present on more differentiated cells. CSCs
and ASCs have many similar properties which may operate through similar molecular pathways, albeit aberrantly in CSCs.

### 1.3.2.2 Source of Cancer Stem Cells

There appears to be several sources from which CSCs can arise. They may arise from normal ASCs, from more restricted progenitor cells, or even from differentiated cells (Fig 1.5) [139, 142]. Normal SCs are the likely targets of mutagenesis leading to the formation of CSCs as they already possess active self renewal pathways, whereas induction of self renewal genes is required to transform more differentiated cells. Further, normal SCs are the only cells with a lifespan long enough to accumulate all the genetic mutations leading to tumourigenesis [142]. To date, CSCs and their ASC counterparts have similar surface marker phenotypes [143], lacking differentiation markers, but it is unclear if these markers are related to SC functions. Finally, some CSCs have been located in the same region as their normal ASC counterparts, and it is possible that niche signals attract the CSC to the niche rather than the CSC arising from the normal ASC [141]. The ASC niche is composed of one or more cells that protect and maintain the ASCs in a quiescent, relatively undifferentiated state. Niche cells sense the environment and signal ASCs to divide when new tissue cells are required [154]. It is possible that niche cells receive the first genetic mutations for tumourigenesis, sending aberrant signals to the resident ASC, conferring CSC properties. It has been hypothesized that CSCs arising from normal SCs result in more aggressive cancer phenotypes, whereas those arising from progenitor cells are less aggressive, though this remains to be proven [139].

### 1.3.2.3 Cancer Stem Cell Markers

Cells express a variety of markers on their cell surface. The expression or absence of these markers has been used to isolate subpopulations of cancer cells for examination of CSC properties. Some cell surface markers including, CD133 (PROM1), CD44, CD24, and THY1 (Table 1.1) are common to several cancers [139]. Investigations into solid tumour CSC markers is required as markers in some tumours have only been examined by a single laboratory, different laboratories have found different markers for the same tumour types, and a potential CSC marker is not always expressed in every individual case of the cancer [139, 155]. The exact function of these markers is yet to be determined. They may be involved in essential SC functions such as self renewal, although this is unlikely given that
they are also present on more differentiated cells [139]. Other intracellular markers have been useful in confirming CSC activity of a cell surface marker defined subpopulation of cancer cells. An example is MUSASHI-1, which appears to mark ASCs in the intestinal and neural systems. MUSASHI-1 may be useful as a confirmatory marker in subpopulations of cells obtained using specific cell surface markers [156].

1.3.2.3.1 CD133 as a Cancer Stem Cell Marker

A commonly investigated potential CSC marker is CD133 or Prominin-1, a 120kDa five transmembrane domain cell surface glycoprotein that may be involved in cell-cell interactions and mature organ homeostasis [157-159]. CD133 has been identified on 0-42.1% of human colon, ovarian and prostate cancer cells, specifically localising to the luminal surface of colon cancer epithelial cells [158, 160-166]. Non-adherent colon cancer cells cultured as spheres expressed CD133 and generated tumours when transplanted into NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice, even after 1 year of sphere culture [150, 158]. It is not clear if these spheres arose from CSCs or progenitor cells, however, when the spheres differentiated they stopped expressing CD133 and lost their ability to form tumours in vivo [150, 158]. Further, CD133⁺ ovarian cancer cells were more efficient at forming clones and proliferated more extensively than the CD133⁻ population [160]. Also, CD133⁺ cells from the ovarian cancer cell lines, A2780
Table 1.1 Common human cell surface cancer stem cell markers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Additional Markers</th>
<th>Tumour type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44⁺</td>
<td></td>
<td>Head and Neck</td>
<td>[158]</td>
</tr>
<tr>
<td>CD24⁻/⁺</td>
<td></td>
<td>Breast</td>
<td>[159]</td>
</tr>
<tr>
<td>EpCAM⁺</td>
<td></td>
<td>Colon</td>
<td>[160]</td>
</tr>
<tr>
<td>CD24⁻ESA⁺</td>
<td></td>
<td>Pancreas</td>
<td>[161]</td>
</tr>
<tr>
<td>EpCAM⁻</td>
<td></td>
<td>Breast</td>
<td>[162]</td>
</tr>
<tr>
<td>CD49hi</td>
<td></td>
<td>Glioblastoma</td>
<td>[163]</td>
</tr>
<tr>
<td>CD133⁺</td>
<td></td>
<td>Medulablastoma</td>
<td>[163]</td>
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<td></td>
<td></td>
<td>Colon</td>
<td>[165]</td>
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<td></td>
<td></td>
<td>Pancreas</td>
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<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>[167]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian</td>
<td>[168]</td>
</tr>
<tr>
<td>ALDH1⁺</td>
<td></td>
<td>Breast</td>
<td>[169]</td>
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<tr>
<td></td>
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<td>Colon</td>
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<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>[171]</td>
</tr>
<tr>
<td>CD90⁺</td>
<td></td>
<td>Liver</td>
<td>[172]</td>
</tr>
<tr>
<td>ABCB5⁺</td>
<td></td>
<td>Melanoma</td>
<td>[173]</td>
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<tr>
<td>SP</td>
<td></td>
<td>Mesenchymal</td>
<td>[174]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian</td>
<td>[175]</td>
</tr>
</tbody>
</table>

and PEO1, were more resistant to chemotherapeutic drugs, upregulated antiapoptotic developmental genes and downregulated death cascade genes, in comparison to the CD133⁺ population [167]. As few as 100 CD133⁺ cells isolated from primary and metastatic colon and brain cancers, and ovarian cancer cell lines, initiated serially transplantable tumours with a similar histoarchitecture and differentiated into CD133⁺ and CD133⁻ cells in a similar ratio as the parent tumour [158, 164, 167, 168]. In contrast, many more CD133⁻ cells were required to initiate tumours, which formed at a much slower rate and were smaller than those initiated by CD133⁺ cells [158, 164, 167, 168]. Importantly, not all CD133⁺ cells were able to initiate tumourigenesis [164] and not all cancers expressed CD133, indicating that it may not mark the CSC in all types of cancer [159, 161-
In some cases of colon cancer CD133 was correlated with more advanced stage tumours, suggesting that it may be a marker for metastasis [162], however, in ovarian cancer the expression of CD133 decreased with metastasis or was unrelated to tumour stage [159, 160]. Further studies are required to determine if CD133 is associated with CSCs or other tumour prognostic factors in other cancers, including other gynaecological malignancies such as endometrial carcinoma.

**1.3.2.3.2 CD44 as a Cancer Stem Cell marker**

The hyaluronin receptor CD44 is a surface glycoprotein with many signalling functions [151, 157], that appears to be expressed on populations enriched for CSCs in human breast, prostate, ovarian and colon cancers. Similar to CD133, spheres generated from unsorted ovarian and colon cancer cells, and those from prostate cancer cell lines, expressed CD44 [148, 150, 151]. These spheres were capable of undergoing self renewing divisions *in vitro* and expressed other SC pluripotency markers including, *OCT-4, NANOG* and *BMI-1* [148, 150, 151]. With differentiation, the spheres lost their expression of CD44 [150], indicating that these might have represented CSCs. Further, *in vitro* evidence for CD44 as a marker for CSCs has been found in colon cancer and prostate cancer cell lines, where the CD44$^+$ population was more efficient at forming large clones [151, 161]. *In vivo*, the CD44$^+$ population in breast, prostate and colon cancers were more efficient at producing larger, more aggressive, serially transplantable tumours in NOD/SCID mice that recapitulated the original tumour phenotype, in a shorter time frame, than the CD44$^-$ population, [139, 161, 163, 169]. However, studies have found a positive correlation with CD44 expression and colon tumour size, and an increase in CD44 expression with increased malignancy in a number of cancer cell lines, which may explain this association in the transplanted mice [151, 162].

Some CSCs have been identified in different and sometimes non-overlapping subpopulations. Human breast CSCs have been identified in the CD44$^+$CD24$^{low}$ population and the ALDH$^+$ fraction [139, 169, 170]. Both sets of cells produced tumours *in vivo* that could be serially transplanted and recapitulated the original tumour phenotype [169, 170]. Interestingly, only 0.1-1.2% of the cells overlapped and these cells were highly tumourigenic [170]. The reason for this distinction may be because the investigators used different tumour subtypes or focused on metastatic, rather than primary breast cancers [169]. Alternatively, the combined ALDH$^+$CD44$^+$CD24$^{low}$ population may represent the
true CSC, with the ALDH$^+$CD44$^+$CD24$^{−/low}$ and ALDH$^+$non(CD44$^+$CD24$^{−/low}$) populations representing more differentiated progenitors that retain some capacity to proliferate in vivo [170]. Similarly, the CD133$^+$ and Lgr5$^+$ fractions appear to locate to different subpopulations in mouse colon cancer [171]. The differences in CSC sub-populations both within the same tumour and between tumours may be related to the effect of culture on the cells, the types of cancers examined (metastatic vs. primary), the ethnicity of the patient source, sorting technologies (magnetic activated cell sorting [MACS] vs. fluorescent activated cell sorting [FACS]), and location of the xenograft (orthotopic vs. heterotropic).

### 1.3.2.4 Cancer Stem Cells and Metastasis

Cancer cells that have not developed into new metastatic growths have been noted at distant sites from the original tumour. This may be because they have not had time to establish a tumour, the efficiency in their removal by the immune system, or their intrinsic lack of tumour initiation capability [142, 149, 172, 173]. There are two theories regarding metastasis and the CSC hypothesis. The first is that the CSC is the only cell with the traits required to establish a tumour at another site and thus it needs to escape the original tumour to establish metastasis elsewhere. Evidence for the role of CSC in establishing metastasis comes from breast cancer studies, where metastatic cells expressing the CSC phenotype, CD44$^+$CD24$^{−/low}$, formed self-renewing mammospheres, differentiated into gland-like structures in soft agar, and gave rise to differentiated progeny including CD44$^{low}$CD24$^{high}$ cells at a higher efficiency than non-metastatic cells [169, 174]. The second theory postulates that CSCs and metastatic cells are different, and that the metastatic cell travels to a distant site to establish a niche and then signals the CSC to travel and take residence in the niche at the new site to initiate a metastatic tumour. This is evidenced by the increased number of CD24$^+$ cells in distant metastasises of human breast cancer [139]. It has also been suggested that the CD44$^+$CD24$^{−/low}$ cells metastasize and then alter their phenotype in response to the new environment. Further studies are required to determine which, if either or both, hypotheses are true.

### 1.3.2.5 Cancer Stem Cells as Prognostic Indicators

While the CSC model is still controversial [175], it is important to note that other cells may also play key roles in the development and progression of cancer [139, 141]. If CSC surface markers relate to functional characteristics, it seems likely that the same cancer
subtypes with similar molecular characteristics between patients will be initiated by similar populations of CSCs. A correlation between putative SC markers and poor prognosis has been observed in some cancers [139, 151, 166, 170, 176], however, no statistically significant correlation between progression free survival and CSC number has been observed in breast cancer [177, 178]. Further investigations are required to determine how marker expression relates to CSC numbers, and how CSC numbers affect tumour progression prognostics.

1.3.2.6 Cancer Stem Cells as Targets for Novel Therapeutics

In cancers with strong evidence for CSCs, new targets are available for drug development that focuses on eradicating the CSC to prevent recurrence. Combining these drugs with traditional cytotoxic agents targeting the remaining cancer cells [178] will prevent them mutating, acquiring self renewal properties and becoming CSCs themselves. Targeting the CSC may be especially important in higher grade and advanced tumours, where evidence indicates that the frequency of CSCs is greater than in lower grade and lower staged tumors [139, 141], and also in tumours where the majority of tumour cells have already been eradicated [178]. It will be important that new treatment options spare normal SCs either in function or delivery.
1.4 Endometrial Cancer

1.4.1 Incidence

Endometrial cancer (EC, Fig 1.6) is the most common gynaecological malignancy affecting women of Australia and the western world [179, 180]. It was the 6th most common cancer in Australian women in 2005 [179] and the 4th most common cancer in American women in 2006 [180]. Two more publicised gynaecological cancers, ovarian and cervical cancers, were ranked 9th and 13th respectively1 in Australia [179]. In 2005, in Australia 1,706 new cases were diagnosed, resulting in 225 deaths and in 2010, 1,909 women are expected to be diagnosed with, and 274 to die from, EC [179]. While this only represents 3.8% of total cancers in Australia, EC is expected to increase in incidence over the next few years, whereas cervical and ovarian cancers are predicted to decrease or remain stable in incidence, respectively [179]. This is because risk factors for EC including, obesity, diabetes, hypertension, nulliparity and age are expected to increase in coming years [75, 181-183]. The 10 year survival rate for EC is approaching 80%, mainly because diagnosis occurs at an early stage due to the readily detectable symptom of abnormal uterine bleeding in post and peri-menopausal women [179, 184]. Approximately 90% of cases are sporadic and the remaining 10% are associated with hereditary diseases such as hereditary non-polyplloid colorectal cancer (HNPCC) [182]. Interestingly, in Japan the rate of EC is 5-10 per 100,000 women annually and women present with primary cancers, whereas 20 per 100,000 American women present with more advanced disease annually [184, 185]. This may be related to the fact that cancer is more common in the western world when compared to Asian countries [186].

1.4.2 Histological Classification of Endometrial Carcinomas

There are a number of histological types of EC; endometrioid carcinoma (adenocarcinoma, adenocarthoma [adenocarcinoma with squamous metaplasia], adenosquamous carcinoma [mixed adenocarcinoma and squamous cell carcinoma]), mucinous adenocarcinoma, papillary serous adenocarcinoma, clear cell adenocarcinoma, undifferentiated carcinoma,

1 Figures quoted here are actually uterine corpus cancer statistics. EC comprises the majority of tumours in this location. EC alone statistics are not published in Australia.
and mixed carcinoma [187]. HNPCC is characterised by abnormalities in DNA mismatch repair genes that eventually results in microsatellite instability (MSI) [188]. Women with this genetic susceptibility have a tenfold higher lifetime risk of developing EC in comparison to the general population [188].

The International Federation of Gynaecology and Obstetrics (FIGO) has classified EC into 3 grades on the basis of morphology (Fig 1.7) [189]. Grade 1 tumours are neoplasms with ≤5% nonsquamous component or those with a nonmorular solid growth pattern (Fig 1.7A) [187, 189, 190]. In grade 2 tumours 6-50% of the neoplasm is nonsquamous and nonmorular (Fig 1.7B), and in grade 3 tumours >50% of the tumour is a solid mass (Fig 1.7C) [189, 190]. In general, grade 1 tumours are well differentiated, grade 2 moderately differentiated and grade 3 poorly differentiated [187]. FIGO grading increases by 1 when nuclear atypia appears inappropriate for the architecture of the grade (e.g. grade 1 becomes grade 2), and when adenocarcinomas contain squamous differentiation the cells are graded according to the nuclear grade of the glandular component [187]. Serous, clear cell and pure squamous cancers are designated grade 3 by convention due to their poor prognosis (Fig 1.7D-F) [190]. While staging (Section 1.4.7) is a major factor in predicting survival rates, different grades within a stage also contribute. Women with stage I or II EC diagnosed with grade 1 tumours have a 5 year survival rate of 93%, for grade 2, 85%, and 69% for grade 3 tumours [187, 190].
32

1.4.3 Types of Endometrial Carcinoma

1.4.3.1 Type I Endometrial Carcinoma

In 1983 Bokhman performed a large study over 20 years and classified sporadic ECs into two types based on their aetiology and clinical behaviour [76, 182, 183]. This classification has been confirmed on the basis of genetic mutational differences between the 2 types. Type I ECs often have endometrioid histology (Fig 1.7A-C) and account for 70-85% of sporadic cases [76, 182, 187]. They have a favourable prognosis as they are often diagnosed at an early stage and are of a low grade [76, 182, 183, 187]. These tumours often arise from endometrial hyperplasia in a setting of unopposed oestrogen in peri- and post-menopausal women, and commonly express oestrogen and progesterone receptors [76, 182, 183]. Mutations in PTEN, β-CATENIN, K-RAS and MSI are molecular markers associated with this type of EC (Fig 1.8) [76].

1.4.3.2 Type II Endometrial Carcinoma

Type II tumours account for 10-20% of sporadic ECs and often have serous papillary or clear cell histology (Fig 1.7D-F) [182, 183, 191]. They tend to be composed of markedly atypical cells that grow in papillary, glandular, or solid patterns [76]. Type II tumours arise
Figure 1.8 Hypothesised roles of Cancer Stem Cells in the Development of Endometrial Carcinoma. (A) Normal endometrial gland with a potential epithelial stem/progenitor cell located at the base of the gland. (B) Epithelial progenitor cell acquires genetic mutations resulting in the development of the cancer stem cell (CSC) in either Type I (left, orange) or Type II (right, dark blue) endometrial carcinoma (EC). Expansion of the CSC clone producing heterogeneous neoplastic epithelial cells in Type I (C) and Type II (D) EC, comprising a small number of CSCs (C, orange; D, dark blue) and numerous differentiated tumour cells (C, light orange; D, light blue).

in a background of atrophic post menopausal endometrium independent of oestrogen and may be preceded by endometrial intraepithelial carcinoma (EIC) [76, 182, 183, 191]. EIC is composed of cells that are indistinguishable from carcinoma cells, but are confined to the endometrial epithelium without invading the underlying stroma [76, 191]. Type II tumours have a poor prognosis as they tend to spread from the site of origin early in the development of the disease [182, 183, 191]. Mutations in TP53 and HER-2/neu genes and
aneuploidy are commonly found in Type II cancers (Fig 1.8), whereas mutations in K-RAS, PTEN or MSI are relatively uncommon, suggesting different molecular pathways for tumourigenesis in the two types of EC [76].

1.4.3.3 Molecular Characteristics of Endometrial Carcinoma

Microarray analysis of EC tissues has strengthened the binary separation of the types of EC, demonstrating that papillary serous carcinomas (Type II EC) are genetically distinct from endometrioid ECs (Type I), with the two cancer types displaying differential expression of over 1000 genes [181]. Further analysis of both types of EC has produced a new set of data where EC is separated into two “clusters” based on the expression of 29 genes [192]. Cluster 2 samples were more aggressive, had a higher stage and grade, increased mitoses and vascular invasion, and while almost all of the Type II samples were in this cluster, almost a 1/3 of the Type I tumours were also included [192]. Interestingly, the Type I samples within cluster 2 had more vascular invasion, necrosis, and mitoses than those in cluster 1 [192].

While clear cell carcinomas are considered a Type II EC, recent evidence suggests that they do not fit simply into the dualistic model of EC [76]. Clear cell carcinoma accounts for 2-3% of ECs and is associated with a 5 year survival rate of 21-75% [76, 193]. Clear cell carcinoma is often associated with serous or endometrioid cells and this may account for the different survival rates [76]. Pure clear cell carcinomas do not show PTEN or TP53 mutations [76]. Immunohistochemical studies have demonstrated that clear cell carcinomas are distinct from endometrioid adenocarcinomas as they exhibit higher cyclin A and reduced cyclin E immunostaining [193]. These proteins are involved with cell cycle regulation and indicate that different mechanisms for cell cycle progression occur in these tumours [193]. Also, mixed histological clear cell types show identical mutations in the clear cells and serous or endometrioid cells, suggestive of a monoclonal origin of the tumour, again suggestive of a CSC origin for EC [76].

There are also uterine carcinosarcomas and mixed müllerian malignant tumours (MMMTs), which are epithelial derived tumours (carcinomatous portion) that have in part differentiated into a stromal sarcomatous cancer [75, 181, 194, 195]. MMMTs have an annual incidence of < 2 per 100,000 women, with a five year survival rate of 25-30% [75, 194]. The stromal portion of these tumours expresses epithelial markers and when
epithelial clones are subcloned they gave rise to epithelial and stromal sub-clones, indicating that the tumour arose from an epithelial cell, potentially a CSC [75]. It is possible, though unlikely, that these tumours arise from the fusion of stromal- and epithelial-derived malignancies, as the lesions are confined to a single region of the uterus. This would require two different spontaneous cancers to occur in the uterus at similar times. As the origin of these tumours appears epithelial they represent a unique model in which to study epithelial-mesenchymal transition (EMT). It has been hypothesised that a loss of E-cadherin in EC is a mechanism whereby epithelial cells metastasise [195]. In a recent study the mesenchymal elements of uterine carcinosarcomas were found to be E-cadherin negative, indicating that the epithelial cell establishing this portion of the tumour had lost its cell-cell adhesions and cell anchorage capabilities [195]. cDNA microarray analysis comparing MMMTs, endometrioid ECs, papillary serous carcinomas and clear cell carcinomas revealed over 5000 genes differentially expressed between MMMTs and endometrioid ECs, and over 1000 when MMMTs were compared to papillary serous carcinomas [181]. This result indicates that the mechanism of tumourigenesis and the molecular behaviour of the carcinomas are likely to be different, but these mechanisms are only starting to be elucidated.

1.4.4 Premalignant Lesions Associated with Endometrial Cancer

Endometrial hyperplasia is often associated with EC. Endometrial hyperplasia is a proliferation of the glands, leading to an increase in the epithelial gland to stroma ratio [76]. It is often associated with unopposed oestrogen stimulation [76]. If left untreated, studies have found that 5-52% of hyperplasias without atypia, and 5-45% of endometrial hyperplasias with atypia, will increases in glandular complexity and degree of cytological atypia eventually being classified as EC [76, 196-199]. The cellular morphology of atypical endometrial hyperplasia is virtually indistinguishable from EC cells, with the lack of epithelial invasion into the underlying stroma as the distinguishing feature of atypical hyperplasia [184].
1.4.5 Genetic Lesions in Endometrial Cancer

1.4.5.1 PTEN

The *PTEN* tumour suppressor gene is the most frequently altered gene in EC (40-83%), specifically affecting more endometrioid (Type I) than non-endometrioid (Type II) ECs [76, 182, 191, 199-201]. *PTEN* is located on chromosome 10q23.3 and undergoes loss of heterozygosity in 20-33% of ECs [76, 182, 191, 201]. It is a lipid and protein phosphatase involved in cell cycle regulation of the G1/S checkpoint, apoptosis, and focal adhesion formation in cell spreading and migration through inhibition of PI3K [76, 182]. Thus, a loss of function of PTEN results in aberrant cell growth, excessive proliferation, and ability to escape apoptosis [76, 182, 191]. Alterations in PTEN expression have been found in 15-75% of hyperplasias, with and without atypia, suggesting that mutations in *PTEN* are an early event in the pathogenesis of EC [76, 182, 191, 199-201]. However, these results should be interpreted with caution as up to 40% of normal proliferative endometria can contain PTEN null-glands due to loss or inactivation of the *PTEN* gene [191, 200], yet only around 2% of women with PTEN-null glands progress to cancer [200]. As it is unethical to leave endometrial hyperplasia untreated, it is impossible to determine the exact number of women who will progress to cancer. This also demonstrates that a “single (molecular) hit” is not sufficient to give rise to a tumour. Interestingly, PTEN-null glands were often located near or next to glands with normal PTEN expression, suggesting that glands are clonal and that the PTEN-null cells proliferate to perpetuate mutant glands [200]. This evidence suggests that inactivation of the *PTEN* gene occurs in a long-lived stem-like cell, favouring the concept of a CSC [74]. In the endometrium, oestrogen stimulates *PTEN* expression and thus PTEN-null glands may have an insufficient response to oestrogen, continuing to proliferate and evade apoptosis [191], although, normal ER and PR immunohistochemical expression has been demonstrated in PTEN-null glands [200], indicating that the insufficiency may be downstream of the receptor. Even without oestrogenic stimulation introduction of normal *PTEN* into *PTEN*-deficient EC cell lines results in cell cycle arrest or apoptosis of the cells [76]. Mutations and loss of *PTEN* expression have also been associated with MSI and *K-RAS* mutations, indicating crosstalk between these pathways may lead to the induction of EC [201].

The phosphatidylinositol 3-kinases (PIK3CA) are lipid kinases that are key players in intracellular signalling networks, regulating cell proliferation, growth, transformation,
adhesion, survival, apoptosis and motility [191]. In EC mutations in PI3K are associated with a poor survival [192]. Interestingly, mutations often resulting in overexpression of PI3K are seen in 36% of endometrioid ECs, most commonly in tumours that also possess a PTEN mutation, indicating the importance of the PI3K signalling pathway and its regulation by PTEN in EC [182, 192].

1.4.5.2 DNA Mismatch Repair Systems

MSIs are genetic mutations in the length of short tandem repeats (microsatellites) within DNA [76, 191]. Slippage errors often occur in microsatellites during DNA synthesis due to their repetitive structure, resulting in insertions and deletions, which are normally corrected by DNA mismatch repair enzymes [191, 202]. In 20-45% of sporadic endometrioid ECs, 3% of complex hyperplasias, and 33% of hyperplasias with atypia, part of the DNA mismatch repair system becomes inactive, often via promoter hypermethylation of the hMLH1 gene, allowing DNA mutations in the microsatellites to accumulate [76, 182, 184, 191, 202-205]. As hypermethylation is present in hyperplasia samples, this may be an early event of endometrial tumourigenesis. Many MSI positive patients present at an early stage with an endometrioid histotype [191, 203], and even when taking this into account, MSI is still an independent predictor of favourable outcome for EC [202]. Interestingly, MSI may be a mechanism for PTEN inactivation as 40-86% of Type I ECs have been found to have lesions in both genes [182, 199, 202]. Also, most ECs with MSI have normal p53 expression [202], indicating that these inactivation mechanisms act through different molecular pathways, consistent with the theory that the molecular progression of types I and II EC are different.

1.4.5.3 RAS Mutations

K-RAS is a proto-oncogene located on chromosome 12p12 which has a role in the regulation of cell growth, proliferation, motility and differentiation [76, 184, 191, 206]. The most common mutation in K-RAS in EC is a single base pair mutation in exon 12 which confers constitutive activity of the protein [184, 198, 199, 205-207]. Mutations have been identified in 10-37% of Type I ECs and endometrial hyperplasia samples, again suggestive of an early role in tumourigenesis, and may also be associated with an aggressive phenotype [76, 182, 198, 206-208]. Interestingly, mutations are more frequent with MSI [209], suggesting a combined tumourigenic pathway. Recently RASSF1A,
located on chromosome 3p21.3, another RAS effector thought to be involved in growth inhibition and apoptosis, was found to be hypermethylated in 74-85% of endometrioid ECs, which may increase with increasing EC stage. Fifty percent of endometrial hyperplasias have hypermethylated RASSF1A [206, 209]. However, there have been conflicting reports on RASSF1A promoter hypermethylation in normal cycling and atrophic endometrium [206, 209]. This discrepancy may be because the study that failed to detect hypermethylation examined paired normal endometrial tissue from women with EC [209], suggesting that hypermethylation may be an early event, increasing the susceptibility for malignant transformation observed in the cancer patients [206, 209]. RASSF1A protein also displayed significant down regulation and inactivation compared to normal endometrial tissues when compared immunohistochemically [209]. Immunoreactive RASSF1A is also associated with K-RAS mutations and MSI, providing further evidence for a combined molecular pathway, or that MSI promotes or allows the accumulation of the other mutation(s) [209].

1.4.5.4 E-CADHERIN, β-CATENIN, WNT Pathway

Mutations in the β-CATENIN gene, CTNNB1 located at 3p21, are found in 13-50% of endometrioid ECs resulting in a gain of function of β-CATENIN [76, 182, 191, 205, 210, 211]. Single base pair missense mutations inhibit degradation of β-CATENIN, promoting its translocation to the nucleus where it constitutively activates target genes in 38% of cases [205, 210]. However, this is has also been observed in ECs without CTNNB1 mutations, suggesting that other β-CATENIN degradation pathways are involved in the molecular profile of EC [205, 211]. β-CATENIN is involved in the WNT signalling pathway and maintains cell polarity by interacting with E-CADHERIN, and is also involved with cellular differentiation [76, 182, 191, 210]. Unlike the other genetic alterations, β-CATENIN mutations are rarely associated with MSI or alterations in PTEN and/or K-RAS [182, 211], suggesting a separate pathway for tumourigenesis. Further, no association between β-CATENIN mutation and age, histological grade and/or stage have been described [211]. The majority (62-87%) of serous papillary and clear cell ECs are associated with down regulation of the E-CADHERIN gene, CDH1, located at 16q22.1 [191, 212]. E-CADHERIN is expressed by epithelial cells and is required for tight connections between cells [191]. Reduced E-CADHERIN results in increased cell motility
[182], perhaps providing a mechanism for metastasis. Down regulation of E-CADHERIN correlates with higher stage tumours [191, 213].

1.4.5.5 p53 Mutations

TP53 is located on chromosome 17 and functions as a tumour suppressor gene. DNA damage causes p53 accumulation, which inhibits CYCLIN-D1 phosphorylation of the retinoblastoma gene resulting in cell cycle arrest and promotion of apoptosis through activation of BAX and APAF-1. A loss of normal p53 activity leads to genetic instability and apoptosis inhibition [182, 188, 191]. Mutations in p53 often result in loss of activity, but resistance to degradation and the accumulation of abnormal p53 within the cell also inhibits the action of wild type p53 [182]. TP53 is altered in 71-90% of endometrial serous papillary carcinomas, 24% of clear cell carcinomas, and 75-80% of EICs, suggesting that it may be an early event in Type II tumourigenesis [76, 182, 191, 193]. Generally p53 abnormalities are associated with a high grade, advanced stage and poor prognosis of EC [191].

1.4.5.6 Her2/neu Mutations

HER-2/neu is an oncogene that produces an epidermal growth factor type II receptor involved in cell signalling and regulation of cell growth and differentiation [182, 191, 214]. HER-2 is overexpressed in 18-80% of Type II ECs and is associated with resistance to chemotherapy, metastasis, recurrence, and poor survival [182, 191, 214-216]. The large variation in the degree of HER-2 expression observed may be explained by differences in patient populations, technical methods including antibodies used and interpretation of staining [214], and types of tumours included in the analysis. Alternatively expression may not confer activity, explaining the variation. HER-2 also appears to be overexpressed in serous papillary or clear cell cancers [215]. HER-2 overexpression may provide selective growth advantage as it increases mitogen activated protein kinases, cellular proliferation [191], and is associated with a loss of ER and PR, p16 and p53 expression, which are all independent poor prognostic markers [214].
1.4.5.7 Factors Influencing Mutation Frequencies in Endometrial Carcinoma

Differences in the percentage of samples affected by genetic alterations and respective protein expression levels are likely due to technical issues, including different staining/nucleic acid isolation protocols between studies, as well as different sample sizes (patient and number of sections examined per patient), frozen vs. paraffin sections, age of sections, and differences in the percentage of cells used as the threshold for positive staining for an overall positive outcome [188, 193]. It may also reflect differences in the populations sampled as incidence rates differ between countries (section 1.4.1). However, \textit{K-RAS} mutations are seen in 27% of Japanese EC cases and only 11% of USA patients, while the mortality rates from EC are similar in the two countries [184]. This indicates that EC may arise via different pathways in different genetic backgrounds, accounting for the differing mutational frequencies and rates of EC.

1.4.6 Steroid Hormone Receptors in Endometrial Cancer

In endometrioid ECs, expression of ER\textsubscript{\alpha} is more common than the expression of ER\textsubscript{\beta} and ER\textsubscript{\alpha} expression decreases with increasing tumour grade, whereas depth of myometrial invasion inversely correlates with ER\textsubscript{\beta} expression [217-221]. The ER\textsubscript{\beta}:ER\textsubscript{\alpha} mRNA and protein ratio has been reported to be high in invasive EC and differs from normal endometrium [218, 219, 222]. This suggests a disturbance in this ratio may facilitate the progression of EC. Interestingly ER\textsubscript{\alpha}, but not ER\textsubscript{\beta}, expression has been correlated with a better prognosis, perhaps because a lack of ER\textsubscript{\alpha} expression is associated with poor cellular differentiation [218, 220, 221, 223]. ER\textsubscript{\alpha} expression is highly correlated with PR expression; together they are inversely associated with histological grade, and thus correlate with a better prognosis [217, 218, 220, 221]. PR-A is often overexpressed in EC, which may relate to the increase in epithelial cell mass [218, 219, 224]. Similarly to ER\textsubscript{\alpha}, a loss of PR expression is associated with a poorer prognosis and myometrial invasion in some studies [218, 223]. In a highly malignant EC cell line (Hec50) that does not express ERs and probably not PRs, introduction of PR-B into the cells slows the cell cycle, induces a secretory phenotype, induces apoptosis, and inhibits EC cell invasion [225], all of which are associated with a more favourable prognosis. PR-B expression also decreases with increasing EC stage [218]. Interestingly, a single nucleotide polymorphism in the promoter
region of PR was found in 15% of ECs and is associated with a modestly increased EC risk [224]. This polymorphism did not result in a loss of PR function, but rather increased transcription and the authors theorised that alterations in the resultant PR-A and PR-B isoform balance predisposed affected women to EC [224].

1.4.7 Surgical Staging of Endometrial Carcinoma

The FIGO staging method released in 1988 changed EC staging from a clinical to a surgical staging, which has been adopted by most pathologists [189]. The staging of gynaecological cancers is determined by epidemiological factors rather than scientific rationale and therefore relating scientific discoveries to surgical stages may not be relevant [226]. In 2008, changes to the 1988 FIGO system were made and were released in 2009 [226, 227]. Comparisons between the new and old system are presented in Table 1.2. In the 1988 classification system stage I tumours confined to the uterine corpus were divided into three subgroups. Stage IA tumours were limited to the endometrium, stage IB tumours had invaded no further than the inner half of the myometrium, and stage IC tumours had invaded into the outer half of the myometrium, but not into the uterine serosa [189, 190]. The recently released five year survival rate for all stage I cancers combined was 88% [190], and overall 71% of EC patients are diagnosed with stage I disease [187]. One problem with dividing stage I tumours into 3 substages was the difficulty in assessing superficial myometrial invasion given the irregular and indistinct border between the endometrial-myometrial junction. Consequently, myometrial invasion was probably over-diagnosed [190]. Review of recent epidemiological data revealed that the survival rates for stage IA and IB tumours was comparable (around 90%) and that the probability of recurrence and death increased in women with deeply invasive tumours, mainly classified as stage IC [187, 190]. The new FIGO staging system has combined stages IA and IB to form the new stage IA, where no or less than half of the myometrium has been invaded by the tumour; and the new stage IB replaces the previous stage IC (Table 1.2; Fig 1.9) [226-228].

Stage II tumours (Fig 1.9) were previously divided into Stage IIA (endocervical glandular involvement) and stage IIB (cervical stromal invasion) [189, 190]. This division has been removed and stage II tumours are designated as those invading the cervical stromal, but not beyond the uterus [226-228]. Endocervical glandular involvement (previous stage IIA) is now considered a stage I disease [226]. One presumes this was because many tumours
classified as stage IIA often had a grade 1 histotype and were associated with the same prognostic factors as stage I tumours [190].

Stage III tumours (Fig 1.9) involve the local and/or regional spread of the tumour [226, 228]. In the 1988 system stage IIIA tumours invaded the uterine serosa and/or adnexae or had a positive peritoneal cytology [190]. Spread to the adnexae is associated with high grade, lymphatic invasion, and deep myometrial invasion, resulting in a poor survival rate (37%), compared to women without adnexae involvement [190]. Similarly, positive peritoneal cytology has a recurrence rate of 30% and is often associated with high grade, deep myometrial invasion, and extra-uterine disease, however, only 5-10% of women with extra-uterine metastases have positive peritoneal cytology [190]. It should be noted that positive peritoneal cytology has been removed from the new stage IIIA classification [226, 228]. Stage IIIB tumours involve vaginal metastases [189, 190] and now may also include parametrial involvement [226, 228]. In these patients > 99% present with spread to the lymph nodes or other distant sites and recurrence is relatively common [190]. These tumours are biologically aggressive with a median survival of 1-2 years and a 5 year survival rate of 25% [190]. Like the old staging system, stage IIIC tumours have metastasised to the pelvic and/or para-aortic lymph nodes [189, 190], however, two new subdivisions have been introduced in the new classification system [226, 228]. Stage IIIC1 tumours have pelvic nodes that are positive for the tumours, and stage IIIC2 tumours have positive para-aortic lymph nodes with or without positive pelvic lymph nodes [226, 228]. Stage IIIC tumours were found to be strongly associated with a high grade, deep myometrial invasion, and lymphatic invasion [190]. If the pelvic lymph nodes are the only site of extra-uterine disease the five year survival rate was 68%, but dropped to 25% when the uterine serosa, adnexae, or vagina were involved, or a positive peritoneal cytology was observed [190]. If para-aortic lymph nodes were positive for the disease the five year survival rate dropped to 20-30% [190].

Finally, stage IV tumour (Fig 1.9) classifications have remained the same, with stage IVA tumours invading the bladder and/or bowel mucosa and stage IVB tumours metastasising either intra-abdominally, into the inguinal lymph nodes, or to other distant sites [189, 226, 228]. Overall the five year survival rate for stage IV EC tumours is only 25% [190].

Other related factors independent from tumour staging affect EC prognosis, including increasing age, which correlates with incidence of disease, tumour grade and depth of
myometrial invasion [187]. Nodal and other metastases increase as depth of invasion and grade increase [187]. All of these factors decrease survival probability. However, surgical staging remains an important factor in prognosis. Changes were implemented into the FIGO staging system in response to observations related to staging and survival times. With this new system prognosis should become more accurate, but may still not be relevant to scientific discoveries.

**Table 1.2 FIGO staging.** Adapted from Creasman (2008).

<table>
<thead>
<tr>
<th></th>
<th>1988 FIGO staging</th>
<th>2008 FIGO Staging</th>
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<tbody>
<tr>
<td><strong>Stage I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>Tumor limited to the endometrium</td>
<td>No or less than half of the myometrium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>invaded</td>
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<tr>
<td>IB</td>
<td>Invasion to less than half of the</td>
<td>Invasion equal to or more than half of</td>
</tr>
<tr>
<td></td>
<td>myometrium</td>
<td>the myometrium</td>
</tr>
<tr>
<td>IC</td>
<td>Invasion equal to or more than half of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the myometrium</td>
<td></td>
</tr>
<tr>
<td><strong>Stage II</strong></td>
<td></td>
<td>Tumor invades cervical stroma, but does</td>
</tr>
<tr>
<td></td>
<td></td>
<td>not extend beyond the uterus</td>
</tr>
<tr>
<td>IIA</td>
<td>Endocervical glandular involvement</td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>Cervical stromal invasion</td>
<td></td>
</tr>
<tr>
<td><strong>Stage III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>Tumor invades the serosa of the corpus</td>
<td>Tumor invades the serosa of the corpus</td>
</tr>
<tr>
<td></td>
<td>uteri and/or adnexae and/or positive</td>
<td>uteri and/or adnexae</td>
</tr>
<tr>
<td></td>
<td>cytological findings</td>
<td></td>
</tr>
<tr>
<td>IIIIB</td>
<td>Vaginal metastases</td>
<td>Vaginal and/or parametrial involvement</td>
</tr>
<tr>
<td>IIIIC</td>
<td>Metastases to pelvic and/or para-aortic</td>
<td>Metastases to pelvic and/or para-aortic</td>
</tr>
<tr>
<td></td>
<td>lymph nodes</td>
<td>lymph nodes</td>
</tr>
<tr>
<td>IIIIC1</td>
<td></td>
<td>Positive pelvic nodes</td>
</tr>
<tr>
<td>IIIIC2</td>
<td></td>
<td>Positive para-aortic lymph nodes with</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or without positive pelvic lymph nodes</td>
</tr>
<tr>
<td><strong>Stage IV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVA</td>
<td>Tumor invasion of bladder and/or bowel</td>
<td>Tumor invasion of bladder and/or bowel</td>
</tr>
<tr>
<td></td>
<td>mucosa</td>
<td>mucosa</td>
</tr>
<tr>
<td>IVB</td>
<td>Distant metastases, including intra-</td>
<td>Distant metastases, including intra-</td>
</tr>
<tr>
<td></td>
<td>abdominal metastasis and/or inguinal</td>
<td>abdominal metastases and/or inguinal</td>
</tr>
<tr>
<td></td>
<td>lymph nodes</td>
<td>lymph nodes</td>
</tr>
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</table>
Figure 1.9 Staging of Cancer of the Corpus Uteri and Metastasis. Adapted from Creasman et al. (2006) [187].
1.5 Cancer Stem Cells in Endometrial Carcinoma

Given that a rare population of epithelial stem/progenitor cells has been identified in human endometrium it is possible that these or their progeny may be the source of the putative CSC that may initiate and maintain EC [34, 74]. The first evidence for a CSC origin of EC was suggested in 1997, in a study on a uterine carcinosarcoma derived cell line [229]. In this study, colony-initiating cells grew for over 50 serial passages and were composed of cells with columnar, small epithelial, moderately sized or large epithelial like, malignant tumour giant and spindle shaped morphologies, similar to those found in the original cell line [229]. These highly proliferative clonal cells expressed immunohistochemical and molecular markers consistent with their parental tissue and recapitulated the tumor phenotype in vitro [229]. They were considered SCs responsible for propagating the cell line [229]. While these results were promising, they were not performed on freshly isolated EC cells, or even a pure EC cell line. At the commencement of my PhD candidature there was no information on the existence of CSCs in human EC.
1.6 Hypotheses

1) That ECs arise from a rare, phenotypically distinct subpopulation of cells within the cancer, cancer stem cells (CSCs), which are responsible for the progression and maintenance of the cancer. Further, that these cells are able to initiate tumours in animal models.

2) That endometrial carcinoma stem cells (ECSCs) possess a range of cell surface markers that differ from those found on other endometrial cancer cells.

3) That ECSCs are enriched in the CD133 expressing subpopulation.

1.7 Aims

1) To determine if CSCs exist in Type I and Type II EC by demonstrating that rare cells within these cancers have the CSC properties of:
   - Clonogenicity; the ability to initiate a clone of cells.
   - Self renewal in vitro; the ability of the cell to produce a daughter cell with CSC characteristics.
   - Expression of pluripotency and self renewal genes.
   - Tumourigenicity; the ability to initiate tumour growth in vivo (Chapter 2).

2) To screen freshly isolated human EC cells for potential marker expression using an antibody panel of known and novel specificities (Chapter 3).

3) To determine if the ECSC population is enriched within the CD133 (the top priority antibody identified by the screening process) expressing population by comparing CD133+ vs. CD133− subpopulations of human endometrial cancer cells for the CSC properties of:
   - Clonogenicity,
   - Self renewal in vitro, and
   - Expression of pluripotency and self renewal genes (Chapter 4).

Overall, I aim to provide evidence for CSCs within human EC, and that these cells are phenotypically distinct from the remaining cancer population and enriched within the CD133 expressing EC cell population.
Chapter 2: Evidence for Cancer Stem Cells in Human Endometrial Carcinoma
Chapter 2: Evidence for Cancer Stem Cells in Human Endometrial Carcinoma

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2.1 Declaration

PART B: Declaration for Thesis Chapter 2

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I was involved in designing and performing the experiments, collecting and analysing the data for 10 of the 12 figures and tables (Figures 1-3, Tables 1 and 2, Supplementary figures 1 and 2, and Supplementary tables 1-3). I wrote the manuscript except for the sections on serial transplantation methods and results, and the pathological diagnosis of the transplanted tumours.</td>
<td>75%</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anne Friel</td>
<td>Designed, collected and analysed the data, writing of the methods section for Supplementary table 4.</td>
<td></td>
</tr>
<tr>
<td>Beena Kumar</td>
<td>Pathological diagnosis of transplanted and original tumours. Writing of methods and results related to this.</td>
<td></td>
</tr>
<tr>
<td>Ling Zhang</td>
<td>Designed, collected and analysed the data for Figure 4 and Supplementary table 4.</td>
<td></td>
</tr>
<tr>
<td>Bo Rueda</td>
<td>Writing of the methods and results section related to serial transplantation of tumours. Designed and analysed the data for Figure 4 and Supplementary table 4.</td>
<td></td>
</tr>
<tr>
<td>Caroline Gargett</td>
<td>Conception and design of experiments, and editing of the manuscript. Responsible for final approval of the manuscript.</td>
<td></td>
</tr>
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</table>

Candidate’s Signature

Date

Declaration by co-authors

The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

| Signature 1 | Date 7th Feb 2010 |
| Signature 2 | 2/9/10 |
| Signature 3 | 2/16/10 |
| Signature 4 | 4/3/10 |
| Signature 5 | 4/3/10 |
Evidence for Cancer Stem Cells in Human Endometrial Carcinoma

Sonya A. Hubbard, Anne M. Friell, Beena Kumar, Ling Zhang, Bo R. Rueda, and Caroline E. Gargett

Abstract
Emerging evidence indicates that the highly regenerative human endometrium harbors rare populations of epithelial progenitor cells. In tumors of other regenerative epithelial tissues, rare cancer stem cells (CSC) have been identified that may have originated from normal epithelial stem/progenitor cells. We hypothesized that CSC are responsible for epithelial neoplasia associated with endometrial carcinoma, the most common gynecologic malignancy in women. Stem cell characteristics of single cells isolated from endometrial carcinoma tissue from women aged 62 ± 11.8 years (n = 34) were assessed. Twenty-five of 28 endometrial carcinoma samples contained a small population of clonogenic cells (0.24% [0.18%–0.4%]), with no significant difference in clonogenic efficiency between the three grades of endometrial carcinoma or between endometrial carcinoma and normal endometrial epithelial samples. Isolated endometrial carcinoma cells transplanted under the kidney capsule of immunocompromised mice in serial dilution (2 × 10^0-1 × 10^4 cells) generated tumors in 8 of 9 samples with morphologies similar to the parent tumors. These tumors recapitulated cytokeratin, vimentin, estrogen receptor-α, and progesterone receptor expression of the parent tumor, indicating that tumor-initiating cells likely differentiated into cells comprising the endometrial carcinoma tissue. Individual clones underwent serial clonal subculture 2.3 to 4 times, with a trend of increasing number of subclones with increasing tumor grade, indicating increasing self-renewal with greater malignancy. Clonally derived endometrial carcinoma cells also expressed the self-renewal genes BMI-1, NANOG, and SOX-2. Isolated cells from primary tumors were serially transplanted 3 to 5 times in nonobese diabetic/severe combined immunodeficient mice, showing self-renewal in vivo. This evidence of cells with clonogenic, self-renewing, differentiating, and tumorigenic properties suggests that a CSC population may be responsible for production of endometrial carcinoma tumor cells. [Cancer Res 2009;69(21):8241–8]

Introduction
Endometrial carcinoma is the fourth most common cancer in the United States and the most common gynecologic malignancy (1). The lifetime risk for developing endometrial carcinoma is 1 in 41 (1), with an estimated 40,100 new cases and 7,470 deaths from the disease in the United States in 2008 (1). Of the two types of endometrial carcinoma type I is the most common (85% of cases); type II endometrial carcinoma, however, typically affects postmenopausal women, arising in atrophic endometrium independent of estrogen, and may be preceded by endometrial intraepithelial carcinoma (2). It is associated with mutations in TP53 and HHR20-new genes (2, 4) and has a poor prognosis (2). The survival rate of endometrial carcinoma is 84% at 5 years (1).

As carcinogenic events can be acquired over many years, it is believed that rare adult stem/progenitor cells have a lifespan sufficiently long enough to accumulate the genetic damage necessary to give rise to cancer stem cells (CSC) hypothesized to initiate carcinomas (5, 6). Recently, a small population of endometrial epithelial stem/progenitor cells were identified in normal human endometrium (6, 7) as clonogenic cells (6) and within the side population (8, 9). Dispersed human endometrial cells transplanted under the kidney capsule of immunocompromised mice reconstituted endometrial tissue, suggesting endometrial stem/progenitor cell activity (10). Endometrial carcinoma is characterized by abnormal endometrial epithelial cell proliferation resulting in an elevated epithelial-to-stromal ratio (2). It is possible that human endometrial stem/progenitor cells are targets of carcinogenesis in this tissue (2, 5, 11), acquiring genetic mutations enabling their transformation into CSC likely responsible for initiation, maintenance, and progression of endometrial carcinoma.

CSC were first identified and characterized in acute myeloid leukemia (12) as rare leukemia colony-forming cells that recapitulated the tumor when grafted into mice (5, 12, 13). Recently, rare, phenotypically distinct CSC were identified in human breast, pancreas, brain, colon, and prostate tumors (14–19) that were more efficient at initiating tumors when transplanted into immunocompromised mice, or forming clones, compared with other tumor cells (15–19). Evidence for CSC in an ovarian serous adenocarcinoma, another gynecologic malignancy, was shown when a small number of cells formed spheroids in vitro and produced tumors when transplanted into nude mice (20, 21). A further 17 human ovarian cancer tissue transplanted under the kidney capsule of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice produced tumors phenotypically similar to the parent tumor (21). These studies...
suggest that many cancers develop from a small subset of cells with self-renewal and tumor-initiating properties, which are responsible for maintaining the heterogeneous tumor cell population (22). The incidence and mortality of endometrial carcinoma is expected to increase in the foreseeable future, as mortality has been largely unaffected by early detection and treatment modalities, and risk factors, such as obesity and age, increase in western women (23). It is important to determine if endometrial carcinoma is established and maintained by a CSC population to develop new drug treatment options. Preliminary evidence for the presence of endometrial CSC was established when some cells from the EMTOKA cell line formed clones in vitro (24). In our study, we focus on primary human endometrial carcinoma and show that some tumor cells possess the CSC properties of clonogenicity, self-renewal, differentiation, and tumorigenicity, which may be responsible for the initiation, maintenance, and progression of endometrial carcinoma.

Materials and Methods

Patient samples. Endometrial carcinoma or hyperplasia samples were collected from 34 women (62 ± 13.8 years) undergoing hysterectomy. Tumors were graded by histopathologists and comprised 2 hyperplasias, 14 grade 1, 9 grade 2, 2 grade 2 to 3, and 3 grade 3 tumors (all type I), and 4 type II tumors (Supplementary Table S1). Samples were obtained from the Victorian Cancer Biobank and collected with patients’ written informed consent in accordance with ethics approval obtained from Southern Health Institutional Review Board. Some samples were obtained from patients at Massachusetts General Hospital following written informed consent or as discarded tissue as outlined in the Institutional Review Board approval.

Tissue samples were collected in HEPES-buffered DMEM and Ham’s F-12 (liftnutro) containing 5% newborn calf serum (CSL) and 1% antibiotic-antimyosin solution (final concentrations: 100 μg/ml penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (liftnutro), stored at 4°C, and dissociated within 24 h.

Tissue dissociation to single-cell suspensions. Single cell suspensions were prepared as described previously (6) with minor modifications. Briefly, tissue was manually dissociated into <0.5 mm fragments and then digested with 25 μg/ml collagenase type IV (Worthington Biochemical) and 0.04 μg/ml DNase type I (Boehringer) for 60 to 150 min at 37°C. Occasionally, 0.2 to 0.4 μg/ml trypsin (liftnutro) was used for 15 to 30 min to ensure dissociation into single cells. Cells were filtered through consecutive 40 and 35 μm cell strainers. Leukocytes were removed with anti-CD45-coated Dynabeads (Dynal Biotech). Epithelial cells were not separated from endometrial carcinoma cell suspensions with EpCam Dynabeads because immunochemistry revealed that many epithelial endometrial carcinoma cells did not express EpCam.

Tissue culture and colony-forming assays. Single endometrial carcinoma cells were cultured in DMEM/F-12 supplemented with 10% FCS (HyClone), Thermo Fisher Scientific), 2 mM L-glutamine (Invitrogen), and 1% antibiotic-antimyosin (liftnutro). Cells were cultured at clonal densities (100–500 per cm²) on 2% gelatin (Sigma-Aldrich)-coated tissue culture ware (BD Biosiences) at 37°C in 5% CO₂ in air.

Cloning plates were monitored daily until cell adhesion, to ensure clones were derived from single cells, and examined every 2 to 3 days and medium was changed weekly. After 3 to 5 weeks in culture, plates were fixed in 10% formaldehyde/PBS for 10 min and stained with Harris hematoxylin (6). Clones (>30 cells) were counted on 2 plates per sample and averaged. Colony-forming efficiency was determined as a percentage (number of colonies) / (number of cells seeded) × 100 ref. 6.

RNA isolation and PCR. Self-renewal and pluripotency gene expression was assessed in 10 samples of freshly isolated and delicately derived endometrial carcinoma cells. RNA was isolated using Trizol (Invitrogen) for samples >100,000 cells or otherwise with RNAeasy microkit (Ambion) according to the manufacturer’s instructions. Embryonic carcinoma cell RNA was used as positive controls (gift from Dr. P. Verma). RNA (0.4–0.5 μg) was reverse transcribed to cDNA (SuperScript III reverse transcriptase; Invitrogen, primers; Invitrogen) and 1 μl of the reverse transcriptase reaction products was amplified in 25 μl with primers (10 pmol) for β-CATENIN, BMI-1, CITED2, VAX2, and GATA3 (internal control; Supplementary Table S2) in an Applied Biosystems GeneAMP PCR system 2700. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Embryonic carcinoma products were excised from the gel using QIAquick gel extraction kits (Qiagen) and sequenced.

Tumorigenicity of dissociated single endometrial cancer cells. Procedures performed on animals were approved by the Monash Medical Centre Animal Ethics Committee. Freshly isolated cells from human endometrial carcinoma tissue were transplanted in serial dilutions (2 × 10¹–10⁴ cells) under the kidney capsule of 6- to 8-week-old female NOD-SCID mice anesthetized with ketamine (10 mg/ml) (Bell Laboratories) and xylazine (5 mg/ml) (Bell Laboratories). A 2 cm dorsal incision was made and kidneys were externalized. Isolated endometrial carcinoma cells encapsulated in fibrin gel (50 μg fibrinogen Calbiochem, EMD Biosciences, 2 μl thrombin, 1,000 US units Sigma-Aldrich) were placed under the kidney capsule. Mice were s.c. injected with 100 ng estradiol valerate (Sigma-Aldrich) in 100 μl peanut oil immediately following surgery and fortnightly thereafter. Mice were sacrificed 12 to 16 weeks after implantation and nephrectomized. Kidneys were fixed in 10% buffered formalin overnight, embedded in paraffin, sectioned at 4 μm, and stained with H&E. The sections were examined in a blinded manner by a pathologist (B.S.K.) unaware of the source of transplanted cells. The presence or absence of tumor in the kidneys, morphologic classification and differentiation based on features such as eosinophilic/papillary differentiation, and pattern of the tumor based on glandular architectural complexity/solid components were analyzed. Tumors were graded (International Federation of Gynecology and Obstetrics (FIGO) system) based on the percentage of tumor showing a solid architecture and nuclear grade of tumor cells (25, 26).

Self-renewal in vitro. Two to 6 weeks after seeding, three individual primary epithelial clones (×60 cells). Fig. 1A middle and right) generated from single cells were reseeded to examine for self-renewal. Well-separated individual clones were trypsinized (0.25%) in cloning rings (Sigma-Aldrich) and replated at <10 cells/cm² to generate secondary clones (27). After 2 to 4 weeks in culture, three secondary clones were reseeded to generate tertiary clones. Serial reodicing continued in this manner until cloning activity was exhausted.

Serial transplantation of primary human endometrial carcinoma explants. Procedures performed on animals were approved by Massachusetts General Hospital Institutional Review Board. Primary endometrial carcinoma or tumor explants were processed as described previously (28) with minor modifications. Endometrial carcinoma tissue was minced to 2 mm pieces and dissociated in IBSS (Cambrex)-2% FCS-1 mM EDTA (Sigma-Aldrich) containing 1 mg/ml collagenase type II (Sigma) 0.05% DNase I (Sigma) at 37°C for 1 h. Cells were filtered through a 100 μm mesh filter (BD Biosciences) and washed in PBS, resuspended in ACK lysis buffer (Cambrex) for 30 s at room temperature to lyse RBC, washed in PBS, and centrifuged over Ficoll-Paque PLUS (GE Healthcare Bio-Sciences). The remaining tumor-derived cells were washed with PBS, resuspended in DMEM (Medicel) containing 2% FCS, 1-glutamine (100 units/ml), penicillin (1%), streptomycin (1%), and 2.5 μg/ml amphotericin B (Sigma), and incubated at 37°C, 5% CO₂ in a humidified chamber for 1 h to collect nonadherent endometrial carcinoma epithelial cells. Nonviable cells were eliminated using the Dead Cell Removal Kit (Miltenyi Biotec). Tumor cells were resuspended in 1× PBS/Matrigel and injected into the right dorsal side of NOD-SCID mice. Mice were assessed regularly for tumor formation. Once tumors reached >10 × 10⁴ mm, mice were euthanized. Tumors were removed, and single-cell suspensions were prepared as above, except that IBSS-2% FCS-1 mM EDTA was the wash solution. Mouse cells were depleted using H-2Kd antibodies (BD Biosciences) and MACS separation columns (Miltenyi Biotec) as per manufacturers’ recommendations. Cells were suspended in 1× PBS/Matrigel and resuspended s.c. into NOD-SCID mice.

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Immunohistochemistry. Paraffin-embedded tumor sections (parent tissue) from blocks used in pathologic diagnosis and paired normal endometrial tissue and tumors from transplanted endometrial carcinoma cells were dewaxed before citrate buffer (0.01 M, pH 6.0) antigen retrieval. Sections were blocked with 3% H2O2 (Biotek Pharmaceuticals) followed by DAKO® Protein Block (DAKOCytomation). Primary antibodies cytokeratin (1:100; 180 mg/mL DAKOCytomation), vimentin (1:50; 59 mg/mL Zymed), and proliferating cell nuclear antigen (1:100; 90 mg/mL Novocastra Laboratories) were diluted in 0.1% bovine serum albumin (PBS) and incubated for 1 h at 37°C or overnight at 4°C for estrogen receptors (ER, 1:200; 260 mg/mL DAKOCytomation), progesterone receptor (PR, 1:500; 30 mg/mL Neomarkers), and PTEN (1:300; 1 mg/mL Cascade Biosciences). Negative controls were matching mouse IgG isotypes at the same concentration. Sections were washed with PBS and incubated with secondary antibody (Envision System) and DAKOCytomation for 30 min at room temperature. Staining was visualized using 3,3'-diaminobenzidine chromogen (Sigma), counterstained with Mayer's hematoxylin (Amber Scientific), and analyzed using a Zeiss Axiostar microscope and image acquisition with Zeiss AxioCam IC3 and Zeiss AxioVision Release 4.6.3.

Statistical analysis. Data were analyzed using GraphPad Prism software (version 5). Gaussian distribution was examined with D’Agostino & Pearson omnibus normality tests and Kruskal-Wallis tests were done for multiple cloning efficiency comparisons and Mann-Whitney U tests for comparison between two groups. P < 0.05 was considered statistically significant.

Results

Cloning efficiency of human endometrial cancer cells. The clonogenic activity of single endometrial carcinoma cells was examined to determine if individual tumor cells initiated clones in vitro. In this prospective study, most samples (25 of 31) contained colony-forming units/cells (CFU) cells (Fig. 1A) that adhered within 7 days of isolation and produced clones. One sample per grade of type I samples failed to adhere and initiate CFU. Clones varied in their appearance. The majority were similar to normal human epithelial clones (Fig. 1A, middle and right), some to stromal clones, whereas others comprised epithelial cells with stromal features. The proportions of these clonal morphologies varied between samples but were predominantly epithelial. The cloning efficiency of all endometrial carcinoma samples was 0.24% (median, range: 0.14% - 0.40%) which was not significantly different from normal human endometrial epithelial samples (median, 0.06% range: 0.01% - 0.31% n = 19; ref. 6). Similarly, there was no difference in cloning efficiency between type I endometrial carcinoma (median, 0.02% range: 0.01% - 0.15%) and normal human endometrial epithelial samples (median, 0.01% n = 10). Comparison between type I and II or hyperplasia samples was not possible because of the small sample sizes of the latter two. No significant difference was observed between the grades of type I endometrial carcinoma (0.13% - 0.18%) grade 1 (n = 11) 0.68% - 1.84%, grade 2 (n = 8) and grade 3 (n = 3). P = 0.12, indicating that rare cells have the potential to initiate clones of daughter cells in all grades and both types of endometrial carcinoma, suggesting the presence of CSC.

Clones from individual samples were divided into three groups based on diameter (Supplementary Fig. S1). The majority of clones were small (diameter < 5 mm), which was unaffected by type I endometrial carcinoma tumor grade. Cloning efficiency was unaffected by concurrent adenomyosis (P = 0.1, n = 20), lymphovascular invasion (P = 0.13, n = 23), or myometrial invasion (P = 0.73, n = 23) of the parent tissue but was significantly higher when associated with leiomyoma (P = 0.01, n = 24; data not shown).

Expression of self-renewal genes. BMI1 and β-Catenin are self-renewal genes commonly found in endometrial carcinoma (29-31). Freshly isolated CYTOKERATIN8-expressing endometrial carcinoma and clonally derived epithelial endometrial carcinoma cells expressed BMI1 and β-Catenin, indicating that the clonally derived cells express self-renewal genes and maintain a cancer phenotype in culture (Fig. 1D). SOX2 and NANOG are associated with pluripotency and self-renewal in human embryonic stem cells (32) and were expressed in clonally derived cells and in freshly isolated samples (Fig. 1C, Supplementary Table S2), indicating that cells capable of forming clones have greater self-renewal potential than other tumor cells while maintaining their epithelial cancer phenotype.

Tumorigenicity of human endometrial carcinoma cells. The tumor-initiating capacity of human endometrial carcinoma cells was determined by transplanting freshly isolated single-cell suspensions in serial dilution under the kidney capsule of
NOD/SCID mice. Histologic examination confirmed cells established tumors in 8 of 9 endometrial carcinoma samples. As few as 10,000 cells established tumors in 4 of 8 samples (Table 1), although 1 million cells were required to establish tumors in 2 of 8 samples. Tumor morphology was consistent in all transplants of the same sample. Tumors derived from grade 2 to 3 or type II cells were larger and possessed more extensive tumor growth than those from grade 1 or 2 tumors, extending into pelvic sinus and peritoneal fat deposits. The tumors derived from transplanted endometrial carcinoma cells showed complex crowded, closely packed, focally coalescing atypical glands with focal areas of glandular fusion and cribriform/solid groups in keeping with adenocarcinoma of endometrioid origin (Fig. 2), which was histologically similar to their parent tumor for each type and grade (Fig. 2), although minor variation in morphologic features occurred in some samples (Fig. 2C).

The transplanted tumors expressed differentiation markers typical of endometrial carcinoma (2) recapitulating cytokeratin, vimentin, ERα, and PR expression of parent tumors (Fig. 3; Supplementary Table S3), indicating that surviving tumor-inhibiting cells (TIC) produced differentiated tumor cells comprising the bulk of the xenograft. Heterogeneous immunostaining was occasionally observed between individual transplant and parent tumors; 7 of 8 and 6 of 8 epithelial-like cells were cytokeratin positive and PR positive, respectively. Heterogeneous vimentin staining of epithelial cells was noted in most samples (Fig. 3) as observed previously in endometrial carcinoma (33); however, consistency between transplants and parent tumors was maintained (Fig. 3).

ERα staining was heterogeneous and mainly located in the epithelial cells, but some staining was observed in surrounding stroma (Fig. 3A). The large number of proliferating cell nuclear antigen–stained nuclei indicates that the tumor cells in the transplants and parent tumors were highly proliferative (Fig. 3D). PTEN inactivation is frequently involved in type I endometrial carcinoma and is preceded by monoclonal loss of PTEN expression in individual glands (34). We compared PTEN immunolocalization in the transplants with paired samples of the original parent endometrial carcinoma tumors and normal adjacent endometrium. Normal endometrial tissue showed typical heterogeneity between individual glands and more than half were PTEN null (Supplementary Fig. S2). The parent tumors showed less heterogeneity (Supplementary Fig. S2, right), whereas the epithelial elements in the transplants were PTEN null in all but one case. This homogenous lack of PTEN staining is suggestive of a clonal origin of the tumors. Collectively, these data suggest that the transplanted endometrial carcinoma cells contained a small number of TIC capable of establishing new tumors in a foreign host with similar morphology and marker expression as the original tumor, indicating the capacity of the TIC to recapitulate the original tumor and differentiate in vivo.

**Self-renewal of endometrial cancer CFU.** Self-renewal of endometrial carcinoma CFU was examined by serially cloning individual CFU. Whereas all samples subcloned at least once, indicating that single CFU underwent self-renewing divisions to establish new clones, the majority of endometrial carcinoma CFU subcloned 8 times (Table 2). The replating frequency increased from 2.5 times in hyperplasia (n = 2) to 4 times in the more malignant type II endometrial carcinoma samples (n = 32), although this trend was not significant (P = 0.56; n = 23) samples; 5 groups). This number of serial clonings indicates substantial self-renewal of endometrial carcinoma CFU in vitro (35, 36).

**Self-renewal of endometrial cancer TIC.** Self-renewal was assessed in vitro using a serial transplantation model (28). Primary endometrial carcinoma single-cell suspensions s.c. injected into NOD/SCID mice established tumors in 3 of 5 mice. Serial transplantation of human cells harvested from these tumors developed further tumors for up to five passages regardless of grade, and final serial transplanted tumors expressed the differentiation markers ERα, PR, cytokeratin, and vimentin in a similar pattern to the original parent tumor (Fig. 4; Supplementary Table S4), supporting the hypothesis that a subpopulation of endometrial carcinoma cells has self-replicative capacity.

**Discussion**

This study shows that a small population of tumor cells residing within human endometrial carcinoma tissues are clonogenic and initiate and differentiate into tumors resembling the original tumor when transplanted into a foreign host and self-renew in vitro and in vivo. The population of cells possessing these properties are possible endometrial CSC.

In this comprehensive study of 32 primary endometrial carcinoma tumors and 2 hyperplasias, we provide the only evidence to date for potential CSC across all grades and both types of endometrial carcinoma and its precursor lesion. Although many cancers have shown self-renewal in culture (15, 37–40), this is the only work showing a trend of increasing self-renewal with increasing endometrial carcinoma tumor grade in vitro. Recently, it was shown that cells with in vitro self-renewal properties existed in four higher-grade endometrial carcinoma samples (28). These studies used ≥2.6 million primary human endometrial carcinoma cells to initiate primary tumors. Fewer cells were required in serial transplants, and two quaternary tumors were established with 10,000 cells (28). The present comprehensive study complements and extends this work (28), because as few as 16,000 primary human endometrial carcinoma cells established xenograft tumors in host mice in 50% of cases, indicating that the frequency of TIC present in primary endometrial carcinoma tissues was similar to that of serially transplanted endometrial carcinoma cells. Further, we showed self-renewal capability of endometrial carcinoma cells in vitro by serial cloning. We also showed that clonally derived CYTOKERATIN-8–expressing endometrial carcinoma cells

**Table 1. Frequency of TIC in human endometrial cancer**

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</tr>
<tr>
<td>2 x 10^6</td>
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<td>1/1</td>
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</table>

NOTE: Freshly isolated cells were transplanted under kidney capsules of NOD/SCID mice and tumors were harvested 12 to 16 wk later. The number of tumors/transplants are shown, ND, not done.
expressed several self-renewal genes, BMI-1, β-CATENIN, SOX-2, and XANG. Furthermore, endometrial carcinoma cells could be serially transplanted and continued to develop tumors. Together, these data suggest that a subpopulation of endometrial carcinoma cells are TIC that undergo self-renewing divisions in vivo. Tumors initiated by transplanted endometrial carcinoma cells had similar histology to parent tumors, a feature maintained on serial transplantation, confirming previous data (28) and extending this finding to all grades of type I endometrial carcinoma. Transplanted cells from grade 2/3 and type II parent tumors resulted in more aggressive tumor phenotypes, also evident by strong proliferating cell nuclear antigen staining, whereas grade 1 tumors were mainly confined to the subcapsular location. Our evidence also suggests that the progeny of the few TIC capable of surviving the transplantation procedures may have differentiated into ERα- and PR-expressing cells in a similar pattern to the primary tumor. Vimentin stained both epithelial and stromal cells in this study, similar to previous observations in endometrial carcinoma (35).

It is possible that vimentin-positive epithelial cells are either undifferentiated or are undergoing epithelial-to-mesenchymal transition (41). The cell clones with morphology intermediate between epithelial and stromal cells also suggest epithelial-to-mesenchymal transition in the progeny of clonogenic cells.

CFU activity, self-renewal, and differentiation are properties of CSC, allowing them to generate additional CSC and phenotypically diverse tumor cells (42). Single endometrial carcinoma or endometrial hyperplasia cells initiated clones with a similar frequency and variability as observed in breast cancer, retinoblastoma, myeloma, and some brain tumors (18, 37, 38, 43) and was not different to normal human endometrial epithelial cells (6), suggesting that a small number of endometrial carcinoma cells may be CSC. More differentiated progenitor cells may have contributed to the observed cloning efficiency, as a range of clone sizes were observed. It is possible that CSC only initiated the larger clones in line with the lower frequency. TIC from the more aggressive type II endometrial carcinoma appeared more efficient at initiating tumors in vivo than type I cells, but paradoxically type II tumor cloning efficiency was lower. This may be due to the culture conditions, optimized for normal human endometrial epithelial cells, which resemble type I more than type II tumor cells. TIC have been identified in xenografts from a range of human cancers, including endometrial carcinoma in this study (15, 20, 29, 37, 38, 41). Most required a minimum of 10,000 to 25,000 transplanted cells to recapitulate tumor immunophenotype (15, 17, 19, 38, 44), and in some, 1 million cells were required (41, 46). Not all dilutions of transplanted endometrial carcinoma cells gave rise to tumors, and not all cells formed clones or underwent self-renewal, suggesting heterogeneity of endometrial carcinoma cell function and TIC potential, conforming to the CSC hypothesis. Our study shows that the potential CSC identified in endometrial carcinoma have similar properties to those characterized in mammary, colon, pancreatic, and prostate cancers, providing further evidence that CSC play a role in the initiation and progression of malignant human tumors.

One limitation of this study was that endometrial carcinoma cells were xenografted into two ectopic rather than orthotopic sites, which provides a more appropriate niche, but unfortunately cells are expelled from the uterus before they attach (47). The larger number of endometrial carcinoma cells required to establish tumors following s.c. compared with subrenal capsule transplantation is not unexpected given the high frequency of cell death anticipated with s.c. transplantation and suggests that the level of vascularization is an important factor. This ability of endometrial carcinoma cells to adapt to new environments may be considered as another CSC property. Stromal cell or cancer-associated fibroblast contamination may be a second limitation of our studies, as variable proportions of stromal cells were observed in endometrial carcinoma samples. It is possible that nonneoplastic cells act as niche cells promoting tumor growth, and for this reason, we did not remove them. Although cancer-associated fibroblasts/stromal cells may have made a small contribution to the cloning efficiency and promoted tumor growth in vivo, this issue can only be addressed once endometrial carcinoma stem cell markers are identified. A final limitation is the variability of tumor grades within single endometrial carcinoma samples. Pathology reports often noted that endometrial carcinoma tissues were not of uniform grade, which may account for minor variations in marker expression observed between transplants and parent tumors as well as within samples. Further evidence of the heterogeneity of endometrial carcinoma cellular composition is indicated by focal staining for ERα, PR, and vimentin within endometrial carcinoma samples as noted previously (33). Future discovery of markers for endometrial carcinoma CSC will enable their prospective isolation for genomic and molecular characterization. Nevertheless, our study shows evidence for potential CSC in endometrial carcinoma.

Figure 2. Histology of transplant and parent tumors. H&E staining of tumors generated from human endometrial carcinoma cells (MP) shows histologic features recapitulating parent tumors (54). A, type I endometrial carcinoma; grade 1 (A), grade 2 (B), and grade 3 (C, D). E, type II transplants and parent tumors. Bar, 100 μm.
Figure 3. Differentiation marker analysis of transplanted and parent endometrial carcinoma tumors. Immunohistochemistry of representative tumors derived from transplanted cells (A–D, top) and corresponding parent tumors (A–D, bottom). A to C, type I grade 1 (A), grade 2 (B), and grade 3 (C); D, type II transplants and parent tumors. CK, cytokeratin; Vim, vimentin (brown with blue hematoxylin counterstain); K, kidney. Transplants were highly proliferative as indicated by proliferating cell nuclear antigen (PCNA) staining. Arrows, representative ERα or PR nuclear staining (brown). Bar, 100 μm.
Table 2. Serial cloning analysis of human endometrial cancer CFU

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<th>Sample type</th>
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<td>Type II</td>
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Note: Median number of replatings averaged from three clones per patient sample.

Type I endometrial carcinoma commonly arises in a setting of unopposed estrogen, which stimulates proliferation of normal endometrial epithelial cells, increasing the opportunity for them to acquire mutations or epigenetic changes conferring self-renewal ability. Mutations and loss of heterozygosity of the PTEN gene result in PTEN-null glands and commonly occur in type I endometrial carcinoma (34). Rare PTEN-null glands have been observed in normal endometrium and even increase in number under conditions of unopposed estrogen, generating precursor hyperplastic lesions, some of which progress to endometrial carcinoma (34). We observed a lack of PTEN immunoreactivity in most tumors derived from transplanted type I endometrial carcinoma cells, even when the parent tumor comprised both PTEN-expressing and PTEN-null epithelial elements. Together, these observations suggest that the tumors initiated from transplanted cells could be monoclonal in origin and may have been initiated from a TIC.

An increased cloning efficiency was observed in samples from women with concurrent leiomyoma, another estrogen-dependent disease. Associated leiomyoma may represent cases where estrogen concentrations are higher, and the accompanying increased cellular turnover of self-renewing endometrial carcinoma CSC may result in increased numbers of CFU in the tumor. Alternatively, leiomyomas are associated with many connective tissue elements that are hypothesized to contain a reservoir of growth factors implicated in cellular proliferation (48).

In this study, we have shown that some cells within a large number of endometrial carcinoma samples of all grades of type I tumors are capable of forming colonies, have TIC and differentiation capacity, and can self-renew in vitro and in vivo. In the few type II endometrial carcinoma tumors examined, we showed that some cells were clonogenic, self-renewing in vitro and had TIC and differentiation activity. This study lays the groundwork for future studies to identify markers enabling the prospective isolation of endometrial carcinoma CSC required to confirm their existence and role in the development of human endometrial carcinoma. Together with the identification of normal human endometrial epithelial stem cell markers, the development and progression of endometrial carcinoma can be investigated, allowing the identification of potential drug targets selective for CSC but sparing normal endometrial stem cells. Such treatments will be particularly useful for early-stage
endometrial carcinoma candidates where the uterus may be conserved and for late-stage cases where hysterectomy is not curative and current treatments target the bulk tumor cells rather than USC.

Disclosure of Potential Conflicts of Interest

C.E. Gargett gave a lecture and provided expert advice at an internal workshop for MerckSerono. The other authors disclosed no potential conflicts of interest.

References

### 2.3 Supplementary Tables

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<td>SPC</td>
<td></td>
<td>L, MI</td>
</tr>
</tbody>
</table>

A, adenomyosis; AH, atypical hyperplasia; CCEC, Clear cell endometrial carcinoma; CH, complex hyperplasia; EC, endometrial adenocarcinoma; EN, endometriosis; H, Hyperplasia; LCC, large cell carcinoma; L, leiomyoma; Lyl, lymphovascular invasion; MI, myometrial invasion; SH, simple hyperplasia; SPC, serous papillary carcinoma.
### Supplementary Table 2a. PCR primer details

<table>
<thead>
<tr>
<th>Protein</th>
<th>Direction</th>
<th>Primer</th>
<th>Tm</th>
<th>Cycle no.</th>
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<td>ACCCAGGAGAAGGGAGGAGCAGCT</td>
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<td>40</td>
</tr>
<tr>
<td></td>
<td>RVS</td>
<td>CGCCTAAGGTTGTTGATGT</td>
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</tr>
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<td>ACAACCTTCAGCAGCT</td>
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<td>35</td>
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<tr>
<td></td>
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<td>AAGTCTTGCTATTACGAC</td>
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<td></td>
</tr>
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<td>BMI-1</td>
<td>FWD</td>
<td>ATGTGCTTGGCTTTGAGG</td>
<td>59</td>
<td>35</td>
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<tr>
<td></td>
<td>RVS</td>
<td>AGTGGTCTGGTCTGTGAAC</td>
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<tr>
<td>SOX-2</td>
<td>FWD</td>
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<td>40</td>
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<tr>
<td></td>
<td>RVS</td>
<td>TTCTCCCCCCTCCAGTTGC</td>
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<td></td>
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<tr>
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<td>FWD</td>
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<td>40</td>
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<tr>
<td></td>
<td>RVS</td>
<td>CCTAGGTCCTGCGTGTATTAC</td>
<td></td>
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</tr>
<tr>
<td>GAPDH</td>
<td>FWD</td>
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<td>40</td>
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<tr>
<td></td>
<td>RVS</td>
<td>GAGGCATGGCTGTGATGTCTTG</td>
<td></td>
<td></td>
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</table>


### Supplementary Table 2b. RT-PCR expression in freshly isolated and clonally derived cells.

<table>
<thead>
<tr>
<th></th>
<th>CK-8</th>
<th>β-CATENIN</th>
<th>BMI-1</th>
<th>NANOG</th>
<th>SOX-2</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly Isolated Cells</td>
<td>10/10</td>
<td>8/10</td>
<td>6/10</td>
<td>10/10</td>
<td>3/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Clonally Derived Cells</td>
<td>6/6</td>
<td>6/6</td>
<td>5/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

### Supplementary Table 3. Frequency of Marker Expression in Transplanted and Parent Tumors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type I Grade 1 Transplant</th>
<th>Type I Grade 2 Transplant</th>
<th>Type II Grade 3 Transplant</th>
<th>Type II Transplant Parent</th>
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</thead>
<tbody>
<tr>
<td>PR</td>
<td>2/3</td>
<td>3/3</td>
<td>2/2*</td>
<td>1/2</td>
</tr>
<tr>
<td>Vim</td>
<td>3/3</td>
<td>3/3</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Era</td>
<td>2/3</td>
<td>2/3</td>
<td>2/2</td>
<td>1/2*</td>
</tr>
<tr>
<td>CK</td>
<td>2/3</td>
<td>3/3</td>
<td>2/2</td>
<td>1/2**</td>
</tr>
<tr>
<td>PCNA</td>
<td>3/3</td>
<td>3/3</td>
<td>2/2</td>
<td>1/1</td>
</tr>
</tbody>
</table>

The number of positively stained tumors/total number of tumors for each grade and type of EC.

* heterogenous staining of the sample; ** no glands visible in tissue.
<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Cell no injected (s.c.)</th>
<th>Transplant number</th>
<th>Time to tumor formation (wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC1 (Grade 1)</td>
<td>20 x 10^5</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9 x 10^5</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8 x 10^5</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3 x 10^5</td>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1 x 10^5</td>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>EC3 (Grade 2/3)</td>
<td>10 x 10^5</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>10 x 10^5</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>EC4 (Grade 3)</td>
<td>36 x 10^5</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>10 x 10^5</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2.3 x 10^5</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>7.5</td>
</tr>
</tbody>
</table>
2.4 Supplementary Figures

Supplementary Figure 1. Distribution of Clone Sizes by EC Grade and Type. Clones were divided into 1 of 3 groups depending on diameter (Solid bars <5 mm, white bars 5-10 mm, grey bars > 10 mm; mean ± SEM). The percentage of clones in each group did not vary significantly with EC grade (grade 1 (n=9) and grade 2 (n=6), P>0.6). Grade 3 (n=1), Type II (n=2), Hyperplasia (n=1).

Supplementary Figure 2. Expression of PTEN in normal human endometrial epithelial glands, endometrial carcinoma glands and primary xenograft tumors. Immunohistochemistry of representative grade 2 (left panels) and 3 (right panels) type I tumors are shown. (PTEN, Variable or lack of PTEN expression is seen in normal human endometrial tissue (A) associated with cancer, parent endometrial carcinoma tumors (B), and in resultant xenograft tumors (C). Inserts negative controls. Scale bars 100 µM.
2.5 Supplementary Information

Clones from individual samples were divided into three groups based on diameter (Supplementary Fig 1). The majority of clones were small (diameter <5 mm, median 96.29%, range 39.42-100%) compared to intermediate (5-10 mm, 5.56%, 0-38.87%) and large (>10 mm, 0%, 0-27.51%; P<0.0001). The percentage of clones in each group was not significantly different between grade 1 and 2, type I samples (<5 mm P=0.95, 5-10 mm P=0.81, >10 mm P=0.67; n=15).

Coating tissue culture plates with or without fibronectin or gelatin had no affect on CE (P=0.89, n=13), nor the size distribution of the clones (< 5 mm P=0.72, 5-10 mm P=0.77, >10 mm P=0.88), indicating that the proportion of CFU in the samples is independent of external factors.
Chapter 3: Identification of Potential Cell Surface Markers of Human Endometrial Carcinoma Stem Cells
Chapter 3: Identification of Potential Cell Surface Markers of Human Endometrial Carcinoma Stem Cells

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3.1 Introduction

Evidence for cancer stem cells (CSCs) in endometrial cancer (EC) have recently been reported (Chapter 2) [74, 230, 231]. A small population of EC tumour cells were able to form clones, initiate and differentiate into tumours resembling the original tumour when transplanted into a foreign host, possessed the side population phenotype, and were able to self renew in vitro and in vivo [230, 231]. However, these functional assays used to identify CSC activity in EC are retrospective and do not identify the CSC phenotype for prospective isolation.

CSCs may drive the progression and maintenance of EC [74, 230, 232], as has been demonstrated for several other solid epithelial tumours [169, 233]. It is hypothesised that the selective removal or death of CSCs would result in the eventual demise of the cancer, as non-CSCs have limited proliferation and self renewal properties compared to CSCs. Studies in other human cancers have identified cell surface markers that enrich for CSC populations (section 1.3.2.3) [139, 146, 161, 163, 167, 169, 177, 234]. However, to date, no markers have been identified that allow the prospective isolation of endometrial cancer stem cells (ECSCs) for their characterisation, hampering our ability to investigate their role in the development of the disease.

Cells express a number of plasma membrane proteins which may be specific for cells of an organ or a cell type within an organ (e.g. PSA in prostate epithelium), while others are ubiquitously expressed by various cell types in many different tissues (e.g. adhesion molecules). Antibodies with specificity to surface antigens or markers can be used to label different cell types in a complex mixture of cells. Cells can then be sorted into various subpopulations on the basis of expression of certain markers. During this study we used a panel of antibodies with specificity to novel and known surface markers created by Dr Hans-Jörg Bühring (HJB, Tübingen University, Germany) [235-237] using hybridoma technology. As the immunogens included tumour cell lines (Table 3.2) and cell lines transfected with human receptors, some antibodies were hypothesised to have specificity for potential adult stem cells (ASCs), CSCs and in particular, ECSCs.

Cell analysers called flow cytometers are used to quantitate the number of cells expressing specific cell surface markers in complex mixtures of single cell suspensions obtained from dissociated tissues [238]. Further, cell sorters rapidly perform fluorescent activated cell
sorting (FACS) to sort rare populations of cells from these complex cell mixtures on the basis of surface marker expression. FACS has been performed on other human cancers including, colon, breast, and ovarian [139, 167, 169, 233].

The aim of this study was to screen cell suspensions obtained from EC samples with the HJB panel of antibodies of known and novel specificities, and to create a priority list in order to develop multicoloour FACS protocols for subsequent testing of subpopulations of EC cells for CSC properties.
3.2 Methods

3.2.1 Collection of Samples

EC or hyperplasia samples were collected from 23 women (62 years ± 11.4 years) undergoing hysterectomy and were graded by histopathologists at Monash Medical Centre. Patient details are presented in Table 3.1. The samples comprised 3 hyperplasias, 6 grade 1, 1 grade 1-2, 6 grade 2, 1 grade 3 tumours (all Type I), 4 Type II tumours, and 2 tumours of mixed EC type. Samples were collected with patients’ written informed consent in accordance with ethics approval obtained from Southern Health Human Research Ethics Committee C.

Table 3.1 Patient Details.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Grade</th>
<th>Pathological details</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>hyperplasia</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td>hyperplasia</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>32</td>
<td>H</td>
<td>hyperplasia</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>1</td>
<td>endometrioid adenocarcinoma</td>
<td>52</td>
</tr>
<tr>
<td>9</td>
<td>I</td>
<td>1</td>
<td>endometrioid adenocarcinoma</td>
<td>67</td>
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<td>36</td>
<td>I</td>
<td>1-2</td>
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<td>42</td>
</tr>
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<td>19</td>
<td>I</td>
<td>2</td>
<td>endometrioid adenocarcinoma</td>
<td>56</td>
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<tr>
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<td>I</td>
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<tr>
<td>25</td>
<td>I</td>
<td>3</td>
<td>endometrioid endometrial carcinoma</td>
<td>56</td>
</tr>
<tr>
<td>24</td>
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<td>2</td>
<td>endometrioid and clear cell and serous papillary adenocarcinoma</td>
<td>65</td>
</tr>
<tr>
<td>42</td>
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<td>2</td>
<td>serous papillary and endometrioid adenocarcinoma</td>
<td>63</td>
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<td>28</td>
<td>II</td>
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<td>undifferentiated large cell carcinoma w/ mucoid and sarcomatoid</td>
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<td>29</td>
<td>II</td>
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<td>serous papillary adenocarcinoma</td>
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</tr>
<tr>
<td>40</td>
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<td></td>
<td>mixed müllerian malignant tumour</td>
<td>58</td>
</tr>
<tr>
<td>41</td>
<td>II</td>
<td></td>
<td>serous papillary carcinoma</td>
<td>69</td>
</tr>
</tbody>
</table>
3.2.2 Preparation of Single Cell Suspensions from Endometrial Carcinoma Samples

Tissue samples were collected in HEPES-buffered Dulbecco’s modified Eagle medium and Ham’s F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, USA) containing 5% newborn calf serum (NCS; CSL, Melbourne, Victoria, Australia; Bench medium) and 1% antibiotic-antimycotic solution (final concentrations: 100µg/ml penicillin G, 100 µg/mL streptomycin sulphate, 0.25 µg/mL amphotericin B; Invitrogen), stored at 4°C and dissociated within 24hrs.

Single cell suspensions were prepared as previously described (Chapter 2) [101, 230] with minor modifications. Briefly, tissue was manually minced into < 0.5 mm fragments and then digested with 20 µg/ml collagenase type II (Worthington Biochemical, Freehold, NJ, USA) and 0.64 µg/ml DNase type I (Roche Diagnostics, Mannheim, Germany) for 60-150 mins at 37°C. Occasionally 0.2-0.4 µg/ml trypsin (Invitrogen) was used for 15-30 mins to ensure complete dissociation into single cells. Cells were filtered through consecutive 40 µM and 35 µM cell strainers.

3.2.3 Production of the Hybridoma Antibody Panel

To create the hybridomas Dr Hans-Jörg Bühring sensitised mice with an antigen from the panel (Table 3.2) and fused the subsequently activated mouse B-lymphocytes with cells from the myeloma cell line, SP20 [239, 240]. The resulting immortalised B-cells produced monoclonal antibodies with the same specificity to the sensitising antigen the activated B-cells were initially exposed to. The immortalised hybridomas have the capacity to grow indefinitely in culture, producing large amounts of marker specific antibodies. The antigens used to activate the female mouse B-lymphocytes are detailed in Table 3.2 and were the retinoblastoma cell line WERI-RB-1, the human embryonic kidney cell line HEK-293, the megakaryocytic cell line UT-7, the breast carcinoma cell line DU.4475, and NIH-3T3 cells transfected with human receptors [239-244]. Further testing of these unpurified hybridoma supernatants by immuno-labelling on isolated cells from haematopoietic and neural tissues demonstrated that they reacted with haematopoietic and neural stem and progenitor cells, without having reactivity with mature cells in these lineages [239, 241].
Table 3.2 Antibody Panel from Dr Hans-Jörg Bühring.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Cell Line Immunogen*</th>
<th>Dilution#</th>
<th>Isoform</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4C4</td>
<td>CD350 (Frizzled-10 receptor)</td>
<td>HEK-293/huFZD10</td>
<td>75%</td>
<td>mlgG2a</td>
<td>[305]</td>
</tr>
<tr>
<td>1B4C3</td>
<td>Her-3</td>
<td>NIH-3T3/huHER-3</td>
<td>30%</td>
<td>mlgG2a</td>
<td></td>
</tr>
<tr>
<td>24D2E2</td>
<td>CD340 (Her-2)</td>
<td>NIH-3T3/huHER-2</td>
<td>75%</td>
<td>mlgG1</td>
<td>[305]</td>
</tr>
<tr>
<td>293-C3</td>
<td>CD133</td>
<td>HEK-293/huCD133</td>
<td>75%</td>
<td>mlgG2b</td>
<td></td>
</tr>
<tr>
<td>2D1D12</td>
<td>Her-3</td>
<td>NIH-3T3/huHER-3</td>
<td>75%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>48B3</td>
<td>DDRI, CD167a</td>
<td>NIH-3T3/huDDR1</td>
<td>75%</td>
<td>mlgG3</td>
<td>[308]</td>
</tr>
<tr>
<td>4FR6D3</td>
<td>CD334</td>
<td>NIH-3T3/huFGFR4</td>
<td>10%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>51D6</td>
<td>DDRI</td>
<td>NIH-3T3/huDDR1</td>
<td>30%</td>
<td>mlgGM</td>
<td>[308]</td>
</tr>
<tr>
<td>57D2</td>
<td>hNPC</td>
<td>UT-7</td>
<td>10%</td>
<td>mlgG1</td>
<td>[241]</td>
</tr>
<tr>
<td>67A4</td>
<td>CD324 (E-cadherin)</td>
<td>T-47D (breast carcinoma)</td>
<td>10%</td>
<td>mlgG1</td>
<td>[237]</td>
</tr>
<tr>
<td>67D2</td>
<td>CD164</td>
<td>T-47D (breast carcinoma)</td>
<td>30%</td>
<td>mlgG1</td>
<td>[236, 306]</td>
</tr>
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<td>97A6</td>
<td>CD203c</td>
<td>UT-7</td>
<td>30%</td>
<td>mlgG1</td>
<td>[235]</td>
</tr>
<tr>
<td>9A3G9</td>
<td>Breast Carcinoma</td>
<td>DU4475 (breast carcinoma)</td>
<td>10%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>BV10</td>
<td>CD135</td>
<td>BaF3/huFLT3</td>
<td>10%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>CH3A4A7</td>
<td>CD344 (Frizzled-4 receptor)</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>30%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>CUB1</td>
<td>CD318</td>
<td>NIH-3T3/huCDCP1</td>
<td>75%</td>
<td>mlgG2b</td>
<td>[240]</td>
</tr>
<tr>
<td>DOR5A2B</td>
<td>Delta opioid receptor</td>
<td>HEK-293/hu-delta-opioid receptor</td>
<td>30%</td>
<td>mlgG2b</td>
<td></td>
</tr>
<tr>
<td>F9C2C1</td>
<td>Human embryonic kidney</td>
<td>HEK-293 (embryonic kidney)</td>
<td>75%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>P3C4</td>
<td>CD172a</td>
<td>NIH-3T3/huSIRPalpha</td>
<td>30%</td>
<td>mlgG2a</td>
<td>[307]</td>
</tr>
<tr>
<td>W3C4E11</td>
<td>CD349 (Frizzled-9 receptor)</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>75%</td>
<td>mlgGM</td>
<td></td>
</tr>
<tr>
<td>W3D5</td>
<td>Retinoblastoma protein</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>30%</td>
<td>mlgG2a</td>
<td></td>
</tr>
<tr>
<td>WSC4</td>
<td>Retinoblastoma protein</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>75%</td>
<td>mlgG2b</td>
<td></td>
</tr>
<tr>
<td>W6B3C1</td>
<td>CD133</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>75%</td>
<td>mlgG1</td>
<td>[258]</td>
</tr>
<tr>
<td>W7C5</td>
<td>CD109</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>75%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>W7C6</td>
<td>Retinoblastoma protein</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>75%</td>
<td>mlgG1</td>
<td></td>
</tr>
<tr>
<td>W8B2B10</td>
<td>MSC</td>
<td>WERI-RB-1 (retinoblastoma)</td>
<td>30%</td>
<td>mlgG1</td>
<td>[241]</td>
</tr>
</tbody>
</table>

*Cell line transfected with plasmid containing a specific immunogen. #Antibody supernatant diluted to percentage shown (as per C. Tan, unpublished). Ab, Antibody.
3.2.4 Immunolabelling of Samples for Flow Cytometry Analysis

Single colour flow cytometry was used to screen freshly isolated EC cells (n=18) for reactivity to the 26 antibodies. Isolated EC cells (5 x 10^4 to 1 x 10^5) were washed in PBS, blocked with 5% goat serum and incubated in a total volume of 100 µl with 4-26 antibody supernatants in separate tubes (Table 3.2) for 45 mins at 4°C. Matched mouse IgG isotype controls diluted in bench medium were substituted for primary antibodies in control tubes. Antibody supernatants were diluted 10-75% in PBS based on optimisations undertaken by C. Tan using freshly isolated normal endometrial epithelial cells. Following incubation for 45 mins at 4°C, unbound antibodies were removed by washing twice in PBS. Samples were again blocked with 5% goat serum and incubated with the secondary antibody (goat anti-mouse IgG-FITC, Silenus, Vic, Australia; goat anti-mouse IgM-FITC, Southern Biotech, USA) for 45 mins at 4°C. Samples were again washed in PBS and either analysed immediately or fixed in 2% paraformaldehyde for data acquisition within 24 hrs using a MoFlo flow cytometer (DakoCytomation, Fort Collins, CO, USA). Data were then analysed using Summit version 5.0.1.3804 (Dako Colorado, Inc.; USA). Cell debris and erythrocytes were electronically gated out using the combined forward and side scatter histogram (Fig 3.1). Following this a minimum of 2000 events were collected for each sample and the percentage of cells reacting with the antibody, above background levels, was determined (Fig 3.2).

Figure 3.1 Scatter plot of a Typical Endometrial Carcinoma Sample. Forward and side scatter of the incident laser beam represents the size and complexity of the cells, respectively. Debris, small and simple cells (those lacking internal cellular organ complexity e.g. erythrocytes) can be electronically “gated” out. Further analysis is only performed on cells within green region 1 (R1).
Figure 3.2 Typical Histogram Displaying the Number of Cells Reacting with Antibody and Isotype Control. Cells that react with the antibody (black line) and matching isotype control (grey line) are electronically counted. Data are combined and plotted on the histogram. Positive events lie within the green box and are above background levels. The position of the box is adjusted until background levels are < 2% of isotype control events.

3.2.5 Immunohistochemistry of Endometrial Carcinoma Tissues

Immuno-reactivity of EC tissues with the antibody panel was performed on 5 µm frozen sections from 1 grade 1, 3 grade 2, and 1 MMMT (Type II) samples. Upon removal from minus 80°C, sections were allowed to reach room temperature before fixation in acetone for 2 mins. Samples were washed three times in PBS between each step unless otherwise stated. Endogenous peroxidise was blocked with 0.3% H₂O₂ (Orion Laboratories PTY LTD, WA, Australia) in methanol for 10 mins, followed by PBA (Immunon Shandon, USA; 1:1 dH₂O) protein blocking for 10 mins. Without washing sections were incubated with one of 26 primary antibodies (Table 3.2) diluted in 0.1% BSA/PBS (bovine serum albumin; Gibco, Invitrogen, Vic, Australia) for 1 hr at 37°C, or matched mouse IgG isotype controls. Unbound antibodies were removed by washing and sections were incubated with biotinylated secondary antibody for 15 mins (LSAB link, DAKO, USA), followed by a 15 min incubation with Streptavidin-HRP (DAKO). Staining was visualised using DAB chromogen (Sigma-Aldrich), counterstaining with Mayers haematoxylin (Amber Scientific, WA, Aust.), and sections were analysed using a Zeiss AxioSkop microscope. Image acquisition was with Zeiss AzioCam ICc3 and Zeiss AxioVision, Release 4.6.3. Staining was scored as strong, medium, or weak as determined by intensity of staining observed by eye (Table 3.3).
Table 3.3 Classification of Immuno-labelling on EC Cells.

<table>
<thead>
<tr>
<th>Intensity of Staining</th>
<th>Percentage of Reactive Cells</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>&gt; 50%</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td>&lt; 50%</td>
<td>Medium</td>
</tr>
<tr>
<td>Medium</td>
<td>0-100%</td>
<td>Medium</td>
</tr>
<tr>
<td>Diffuse</td>
<td>0-100%</td>
<td>Weak</td>
</tr>
</tbody>
</table>

3.2.6 Statistical Analysis

Antibody expression data were analysed using Microsoft Excel (Microsoft Office, version 2003). Mean percentage positive cells determine by flow cytometry (Fig 3.2) and the variation (Standard deviation, SD) in the percentage positive cells was examined for the different antibodies. As the percentage of positive cells varied several fold between antibodies, SDs were considered as a percentage of the mean percentage positive cells (i.e. SD/mean percentage positive cells x100) to determine a marker’s robustness.

3.2.7 Strategy for Prioritising Panel Antibodies

Prior to the isolation of subpopulations and investigation of CSC properties in EC cells, screening of the cells for reactivity to the HJB antibody panel was required. Immuno-reactivity of EC tissues to the antibody panel by both flow cytometry and immunohistochemistry were compared and arranged into a priority list (Fig 3.3). Antibodies were removed from the list in a systematic way as shown in figure 3.3 using a simple algorithm. Those that did not react were removed first, followed by those that stained the tumour cells homogeneously. Remaining antibody supernatants were compiled into a priority list based on the number of EC cells expressing the antigen, the robustness (variance in expression between samples, section 3.2.6) of the antibody supernatant, and the intensity of EC staining in tissue sections.
**Figure 3.3 Strategy for Creating an Antibody Priority List.** All antibody supernatants were analysed by immunohistochemistry (IHC) and flow cytometry (FC). Any antibody reacting with all endometrial cancer (EC) cells, EC stromal cells only, or to antigens that are sensitive to the isolation process, were excluded. Further, lack of concordance between or within IHC and FC data indicated lack of robustness and consistent expression on potential EC stem cell subpopulations.
3.3 Results

3.3.1 Screening of Human Endometrial Cancer Cells for Immuno-reactivity to an Antibody Panel

Initial screening with the antibody panel was undertaken to determine whether isolated EC cells reacted with the antibodies, to quantify the percentage of reactive cells (percentage positive cells), and to identify those antibodies which should remain on the priority list (Fig 3.3). The number of cells acquired from a single EC sample was highly variable (1-11 x 10^5) and was the limiting factor in the number of antibodies that could be examined in a given sample, ranging from 3-21 antibodies.

EC cells reacted with most of the antibodies (25/26) in the panel, however, this reactivity varied greatly between samples for any given marker (Table 3.4 and Supplementary Table 3.1). The percentage of cells reacting with the antibodies in the panel varied from 0-78%. Only 8 antibodies (57D2, 9A3G9, 24D2E2, 67D2, CUB1, W8B2B10, W3D5, and W5C4) consistently reacted with a proportion of EC or hyperplasia cells on at least 5 patient samples. Conversely, CH3A4A7 (CD344) was the only antigen that was consistently not expressed (< 1%) on EC samples (Fig 3.4A), and thus was considered not useful as a potential endometrial cancer stem cell (ECSC) marker. No differences (P>0.05) in the percentage of positive cells reacting with any of the antibodies from the panel were found between Type I, Type II and hyperplasia samples (Table 3.4 and Supplementary Table 3.1). Interestingly, none of the antibodies tested appeared to distinguish ECs from its precursor lesion, hyperplasia. This could indicate commonality of the cells involved in the pathology, but is probably due to the large variability of reactivity of cells within tumour types. Therefore, the percentage of positive cells reacting with a given antibody for all samples were averaged and used to develop the priority list. The variability (SD) in the number of cells reacting with a given antibody was also considered in the development of the priority list, with those showing the least variability ranking higher on the priority list than those showing wide variation (section 3.3.3).
Table 3.4 Flow Cytometric Analysis Showing the Percentage of Cells Isolated from Endometrial Carcinoma (EC) and Hyperplasia Tissues Cells Reacting with Antibodies in the Panel.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type I and II EC (n)</th>
<th>SD</th>
<th>All Samples (n)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>67D2</td>
<td>67.31 (4)</td>
<td>11.53</td>
<td>70.89 (6)</td>
<td>10.83</td>
</tr>
<tr>
<td>24D2E2</td>
<td>62.21 (3)</td>
<td>9.10</td>
<td>48.94 (4)</td>
<td>1.65</td>
</tr>
<tr>
<td>W5C4</td>
<td>52.22 (6)</td>
<td>16.81</td>
<td>52.22 (6)</td>
<td>16.81</td>
</tr>
<tr>
<td>W6B3</td>
<td>37.42 (4)</td>
<td>23.79</td>
<td>22.82 (7)</td>
<td>21.13</td>
</tr>
<tr>
<td>W3D5</td>
<td>19.00 (7)</td>
<td>16.84</td>
<td>19.00 (7)</td>
<td>16.84</td>
</tr>
<tr>
<td>67A4</td>
<td>18.49 (3)</td>
<td>12.12</td>
<td>11.59 (6)</td>
<td>13.57</td>
</tr>
<tr>
<td>F9C2C1</td>
<td>13.92 (4)</td>
<td>22.05</td>
<td>18.92 (6)</td>
<td>16.43</td>
</tr>
<tr>
<td>184C3</td>
<td>12.95 (2)</td>
<td>11.23</td>
<td>8.84 (4)</td>
<td>17.49</td>
</tr>
<tr>
<td>293-C3</td>
<td>11.50 (7)</td>
<td>7.90</td>
<td>11.50 (7)</td>
<td>7.90</td>
</tr>
<tr>
<td>CUB1</td>
<td>11.19 (4)</td>
<td>9.25</td>
<td>11.92 (6)</td>
<td>9.73</td>
</tr>
<tr>
<td>2DID12</td>
<td>9.39 (4)</td>
<td>6.31</td>
<td>8.31 (6)</td>
<td>6.73</td>
</tr>
<tr>
<td>W8B2B10</td>
<td>7.97 (4)</td>
<td>3.56</td>
<td>6.84 (6)</td>
<td>2.18</td>
</tr>
<tr>
<td>BrCa</td>
<td>7.94 (3)</td>
<td>27.64</td>
<td>31.31 (6)</td>
<td>7.03</td>
</tr>
<tr>
<td>DOR5A2B</td>
<td>7.81 (6)</td>
<td>9.11</td>
<td>7.81 (6)</td>
<td>9.11</td>
</tr>
<tr>
<td>hNPC</td>
<td>7.05 (3)</td>
<td>4.45</td>
<td>6.32 (6)</td>
<td>5.85</td>
</tr>
<tr>
<td>P3C4</td>
<td>6.69 (7)</td>
<td>10.58</td>
<td>6.69 (7)</td>
<td>10.58</td>
</tr>
<tr>
<td>51D6</td>
<td>6.48 (5)</td>
<td>4.92</td>
<td>6.71 (6)</td>
<td>5.46</td>
</tr>
<tr>
<td>48B3</td>
<td>5.30 (6)</td>
<td>7.75</td>
<td>5.30 (6)</td>
<td>7.75</td>
</tr>
<tr>
<td>1/4C4</td>
<td>2.57 (6)</td>
<td>3.99</td>
<td>2.57 (6)</td>
<td>3.99</td>
</tr>
<tr>
<td>BV10</td>
<td>2.28 (7)</td>
<td>3.12</td>
<td>2.28 (7)</td>
<td>3.12</td>
</tr>
<tr>
<td>4FR6D3</td>
<td>2.02 (4)</td>
<td>2.96</td>
<td>1.62 (6)</td>
<td>3.74</td>
</tr>
<tr>
<td>W7C6</td>
<td>1.52 (7)</td>
<td>1.79</td>
<td>1.52 (7)</td>
<td>1.79</td>
</tr>
<tr>
<td>W3C4E11</td>
<td>1.38 (4)</td>
<td>3.35</td>
<td>2.55 (6)</td>
<td>2.03</td>
</tr>
<tr>
<td>97A6</td>
<td>1.23 (4)</td>
<td>6.09</td>
<td>3.80 (7)</td>
<td>2.04</td>
</tr>
<tr>
<td>W7C5</td>
<td>0.46 (7)</td>
<td>0.59</td>
<td>0.46 (7)</td>
<td>0.59</td>
</tr>
<tr>
<td>CH3A4A7</td>
<td>0.06 (7)</td>
<td>0.10</td>
<td>0.06 (7)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Results are means of (n) samples with SD shown. SD, standard deviation. Note that supplementary table 3.1 shows data for type I, II and hyperplasia samples separately.

3.3.2 Screening of Frozen Sections of Endometrial Cancer Tissues for Immuno-reactivity to the Antibody Panel

Flow cytometry quantitated the number of EC cells that reacted with each antibody, but this technique did not distinguish which types of cells were immunoreactive. Immunohistochemical analysis was undertaken to determine whether epithelial, stromal or vascular cells were reacting with the antibodies, the heterogeneity of the staining in the epithelial-like compartment, and to detect whether antigens were enzyme sensitive (section
3.3.3). There was a wide range of immunohistochemical reactivities observed. In general Type I and Type II EC samples displayed differential reactivities to each antibody (Table 3.5 and Supplementary Table 3.2, Fig 3.4D), however, limited conclusion can be drawn from this as only a single Type II sample was available for investigation and expression patterns within the Type I samples also varied for 16 of the antibodies tested (Fig 3.5).

67D2 (Fig 3.4B) was the only antibody that ubiquitously reacted with cells in both Type I and Type II samples, thereby excluding it as a potential ECSC marker. Conversely, 4FR6D3 (Fig 3.4C), 9A3G9, and 57D2 failed to react with any cells in either Type I or Type II EC tissue sections, thereby excluding these antibodies as potential ECSC markers. Another group of antibodies (W7C5, W7C6, W3D5, W5C4, BV10 and W3C4E11; Fig 3.4E) were more selective for stromal cells than epithelial cells in the Type I samples. In the Type II sample the reactivity was absent, patchy or appeared to be perivascular (Fig 3.4E). However, as this was a screen, no detailed tests were performed to determine the exact location of the vessels and any co-localisation. As EC is characterised by an increase in the epithelial to stromal ratio and is an adenocarcinoma, it was hypothesised that the ECSC would be located in the epithelial compartment. Thus the antibodies with stronger stromal reactivity were removed from the priority list. Ideally, a CSC marker should react strongly with a small number of cells. This was not observed with any of the antibodies tested, however, only single tissue sections for each marker were examined, and it is possible that rare CSCs were missed.
Table 3.5 Summary of Reactivity of Human Endometrial Carcinoma (EC) to the Antibody Panel.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>All EC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strong</td>
<td>medium</td>
<td>weak</td>
</tr>
<tr>
<td>67D2</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>293-C3</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>W6B3</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>DOR5A2B</td>
<td>2</td>
<td>2 (d,d)</td>
<td>1</td>
</tr>
<tr>
<td>67A4</td>
<td>2</td>
<td>2 (m,m)</td>
<td>1</td>
</tr>
<tr>
<td>97A6</td>
<td>2</td>
<td>1 (m)</td>
<td>2</td>
</tr>
<tr>
<td>CUB1</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>W3D5</td>
<td>1</td>
<td>2 (d,m)</td>
<td>2</td>
</tr>
<tr>
<td>51D6</td>
<td>1</td>
<td>1 (m)</td>
<td>3</td>
</tr>
<tr>
<td>W8B2B10</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>W5C4</td>
<td>0</td>
<td>5 (d,d,m,m,m)</td>
<td>0</td>
</tr>
<tr>
<td>48B3</td>
<td>0</td>
<td>4 (d,d,m,m,m)</td>
<td>1</td>
</tr>
<tr>
<td>1B4C3</td>
<td>0</td>
<td>3 (d,d,m)</td>
<td>2</td>
</tr>
<tr>
<td>2DID12</td>
<td>0</td>
<td>3 (d,m,m)</td>
<td>2</td>
</tr>
<tr>
<td>1/4C4</td>
<td>0</td>
<td>2 (d,m)</td>
<td>3</td>
</tr>
<tr>
<td>W7C6</td>
<td>0</td>
<td>2 (d,m)</td>
<td>3</td>
</tr>
<tr>
<td>24D2E2</td>
<td>0</td>
<td>2 (m,m)</td>
<td>3</td>
</tr>
<tr>
<td>F9C2C1</td>
<td>0</td>
<td>2 (m,m)</td>
<td>3</td>
</tr>
<tr>
<td>BV10</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CH3A4A7</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>P3C4</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>W3C4E11</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>W7C5</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>4FR6D3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>57D2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9A3G9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data presented are the results from 5 samples and are classified based on Table 3.4. Medium intensities are presented in () with d representing darkly stained cells, and m representing medium stained cells. Highlighted in red are antibodies used in Chapter 4. Note that supplementary table 3.2 shows data for type I and II samples separately.
Figure 3.4 Flow Cytometry and Immunohistochemistry Profiles of Endometrial Carcinoma cells with Representative Antibodies. Some antibodies failed to react with any Type I and most Type II endometrial carcinoma (EC) cells (A), or reacted ubiquitously with EC cells (B) in either Type I (left panels) or Type II (middle panels) samples (Immunohistochemistry [IHC] inserts in left and middle panels). Others possessed few immuno-reactive cells by flow cytometry (FC), and IHC (C), and others displayed differential reactivities between the two types of EC (D). A few antibodies were restricted to tumour stromal cells (E), and some appeared to react with antigens that may be enzyme sensitive and not detected by FC (F). Right panels show percentage positive cells on individual EC samples by FC.
3.3.3 Creation of an Antibody Priority List

Antibodies that may identify potential ECSCs were arranged into a priority list (Table 3.6 and Supplementary Fig 3.1) following the procedure detailed in figure 3.3. The creation of this priority list involved collating the data obtained from both immunohistochemical and flow cytometry investigations (Fig 3.6). Antibodies that did not react with any EC cells by flow cytometry or immunohistochemistry (e.g. Fig 3.4A and C) were removed from the candidate list, as were antibodies that stained only stromal cells (e.g. Fig 3.4E) because EC possesses an epithelial phenotype the CSC is hypothesised to be located within the epithelial-like compartment. Following this, antibodies that reacted homogenously with all tumours cells (e.g. Fig 3.4B), and thus would not discriminate potential ECSC from remaining cancerous cells, were removed from the priority list.

Finally, antibodies with non-concordance between flow cytometry and immunohistochemical results (e.g. Fig 3.4E) were also removed from the list. This included antibodies that stained strongly by immunohistochemistry, but stained few cells by flow cytometry (e.g. Fig 3.4F). After these antibodies were removed from the list the remaining 13 antibodies were ranked from highest to lowest based on the percentage of cells expressing the antibody, the variation in antibody expression between EC samples (robustness), and the pattern of expression by immunohistochemistry. Details of the top three antibodies are shown in figure 3.7.
Table 3.6 Priority List of Antibodies to Test for Potential Cancer Stem Cell Markers in Human Endometrial Carcinoma.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Antibody</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W6B3</td>
<td>CD133</td>
</tr>
<tr>
<td>2</td>
<td>24D2E2</td>
<td>Her-2</td>
</tr>
<tr>
<td>3</td>
<td>293-C3</td>
<td>CD133</td>
</tr>
<tr>
<td>4</td>
<td>67A4</td>
<td>CD324 (E-cadherin)</td>
</tr>
<tr>
<td>5</td>
<td>W8B2B10</td>
<td>MSC</td>
</tr>
<tr>
<td>6</td>
<td>CUB1</td>
<td>CD318</td>
</tr>
<tr>
<td>7</td>
<td>2DID12</td>
<td>Her-3</td>
</tr>
<tr>
<td>8</td>
<td>DOR5A2B</td>
<td>Delta opioid receptor</td>
</tr>
<tr>
<td>9</td>
<td>51D6</td>
<td>DDRI</td>
</tr>
<tr>
<td>10</td>
<td>F9C2C1</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>11</td>
<td>1B4C3</td>
<td>Her-3</td>
</tr>
<tr>
<td>12</td>
<td>48B3</td>
<td>DDRI, CD167a</td>
</tr>
<tr>
<td>13</td>
<td>1/4C4</td>
<td>CD350 (Frizzled-10 receptor)</td>
</tr>
</tbody>
</table>

In red, investigated in chapter 4.
Figure 3.6 Creation of an Antibody Priority List. Endometrial carcinoma cells were analysed for reactivity with antibody supernatants. Antibodies that did not fit the criteria for labelling a potential endometrial carcinoma stem cell were removed. The remaining 13 antibodies were ordered into a priority list as described in section 3.3.3.
Figure 3.7 Expressions of W6B3, 24D2E2, and 293-C3 by Endometrial Carcinoma (EC) Cells. W6B3 (A), 24D2E2 (B), and 293-C3 are ranked 1-3, respectively, on the priority list for potential markers of EC stem cells. Left (Type I EC) and middle (Type II EC) panels are flow cytometry histograms with immunohistochemistry inserts. Immunohistochemistry only shown in the middle of panel B as no flow cytometry profile is available for 24D2E2 reactivity with Type II samples. Right panels, each dot represents and individual sample.
3.4 Discussion

Using a systematic screening approach, EC samples were investigated with a panel of 26 antibodies with the potential to identify ECSC markers. Most antibodies marked a variable number of EC cells, with a range of staining patterns observed. The combination of data created from flow cytometry and immunohistochemistry screening of samples allowed the development of a priority list of antibodies. The antibodies on the priority list can be tested as potential markers for ECSCs using previously developed functional CSC assays (Chapter 2) [230].

This study is the first to investigate the expression of potential surface antigens in EC using a panel of 26 potential ASC marker antibodies. While most of the antibodies (25/26) reacted with a proportion of EC cells, 12 were eliminated as potential ESCS markers. These antibodies reacted homogenously will all EC cells, only with stromal cells, or their epitopes appeared to be enzyme sensitive, producing discordance between immunohistochemistry and flow cytometry immunoreactivity. The remaining 13 antibodies were arranged into a priority list of potential ECSC markers based on the mean percentage of cells expressing the marker, and the markers robustness and consistency of marker expression between samples. Interestingly, the antibody priority list developed for Type I EC cells alone barely changed with the addition of hyperplasia and/or Type II EC results. This may indicate that the cell subpopulations driving these hyperproliferative diseases are similar, involving common cellular pathways. However, it is more likely that the three grades of Type I EC will possess CSCs with similar marker phenotypes and it is possible that cell surface marker phenotypes will differ between the CSCs of both types of EC, and even within the subtypes of Type II EC due to different signalling and molecular lesions [76, 181, 183, 192]. Future testing of the EC sorted cells into subpopulations on the basis of their reactivity to the lead antibodies in functional CSC assays will indicate if they select for ECSCs, in either of one or both types of EC. If these antibodies do select ECSCs, then they can be used to prospectively isolate the ECSC enabling their characterisation on molecular, genetic and epigenetic levels, and their roles in the development and maintenance of the disease can be investigated. On a larger scale these markers may also identify CSCs in other tumours or normal epithelial endometrial stem/progenitor cells.
The main limitation of this study was that not every grade or both types of EC or hyperplasia samples were screened for all markers by flow cytometry or immunohistochemistry. Unfortunately optimisation of each antibody for each type and grade of EC was limited by available human tissue resources. Samples are not common and small, limiting the number of freshly isolated cells or tissue sections available from each sample. Nevertheless, most antibodies were screened on 5-7 different EC samples, providing relatively similar results. Previous studies investigating single potential CSC markers on metastatic breast, higher grade prostate cancer, colon, ovarian, and brain tissue cancers investigated 5-15 individual samples [148, 161, 165, 169, 234]. However, there is no published work indicating how many samples, if any, were screened before functional assays were performed. A further complication was that the pathological details of the fresh EC samples were not available at the time of flow cytometry analysis, which was examined within 24 hours of sample collection. To overcome this limitation, frozen-thawed single cell suspensions of EC cells from known grades and types were investigated. However, large differences in the percentage of positive cells for several markers were observed between freshly isolated and frozen-thawed cells from the same sample, suggesting loss of certain cell subpopulations during the freeze-thaw process. An advantage of our approach is that once a marker is discovered and verified it will be used in a similar prospective manner, i.e. before the tumour is histologically diagnosed, and thus it would be ideal if the marker was robust enough to identify CSCs in both types of EC and their precursor lesions. Finally, dead cells, stromal cells and leukocytes were not removed from the screened population. It is possible that these cells contributed to the percentage of cells reacting with an antibody from the panel. The aim of these experiments was to screen EC cells for reactivity to the antibody panel. Further in depth analysis requires much more time, human EC tissue and antibody resources. These limitations were taken into account when developing the priority list and with more vigorous investigation of the antibodies on the priority list, it is hoped that a potential ECSC marker will be discovered.

In the development of the antibody priority list, certain factors were taken into account. Average percentage of positively reacting EC cells was ranked from highest to lowest, with antibodies reacting with < 1% of cells removed from the list as this result was considered artefact. Such small amounts of reactivity were considered artefact for several reasons. Firstly, we hypothesise that the CSC population will be enriched in a subpopulation expressing a single marker, but that a single marker is unlikely to be highly selective for
only ECSCs. A partially purified population of EC cells can be further analysed for additional markers to increase purity using an iterative process. Secondly, the average percent positive cells were often reduced because one or more samples did not express the antigen, indicating the lack of consistency in expression, another concern in the creation of the priority list. Following this ranking, immunohistochemical analysis was used to refine the flow cytometry screening by identifying cell specific immune reactivity. Markers that only stained stromal cells were removed from the list as EC is a tumour of endometrial epithelial cells [76], and thus it is hypothesised that ECSCs will be of an epithelial origin. Also, markers that ubiquitously marked the majority of EC cells by immunohistochemistry were removed, as they would not prospectively isolate rare ECSCs. Finally, antibodies with strong immunohistochemical staining, but little or no flow staining, were removed from the priority list. It was assumed that these antibodies stained antigens that were enzyme sensitive and were removed during the isolation process. Thus, these antibodies were not useful for subsequent cell sorting approaches using FACS. Ideally, a potential ECSC marker should react with single or small clusters of cells in tissue sections, indicating that the marker has restricted expression and is not present on the more common differentiated tumour cells [164, 176, 234]. This was not observed with any of the antibodies tested, however, the range of EC samples examined was quite limited. By combining all these properties a priority list was developed for the screening of EC cell subpopulations for CSCs properties using functional assays to identify potential ECSC markers (Chapter 4).

The investigation of CSCs and markers that identify these cells are increasingly being reported [35, 161, 239, 245-247]. These investigations commonly identify potential stem cell (SC) surface markers by one of two methods. Firstly, a method for isolating prospective ASCs or CSCs from the organ has been developed which does not rely on the expression of markers. These isolated SCs are tested for the expression of known SC markers [150], or differential protein array comparisons with the remaining cancerous cells could be performed. The alternate method involves testing previously identified SC markers from one tumour or ASC in tumours from another organ, which has been exemplified by the recent focus on CD133 [158, 164, 169, 234, 248, 249]. The advantage of the more comprehensive screen performed in this chapter is that a number of potential SC markers that have not been previously identified have been examined, enlarging the pool of potential SC markers that may be tested in other organs. Further, insight into the
molecular pathways in EC may be determined once the specific antigens for CSCs are discovered and their functions tested.

Previous investigations have demonstrated that the unpurified hybridoma supernatants used in this study reacted with haematopoietic and neural stem and progenitor cells, without having reactivity with mature cells in these lineages [239, 241]. Interestingly, these antibodies react with targets associated with a number of cellular functions. Specifically, the antibodies in the priority list developed in this study react with targets that are involved in cellular growth, differentiation, adhesion, and even analgesia [237, 240, 241, 305]. Exactly how each target functions in the potential SC is still to be determined, and may be investigated once ECSCs can be prospectively isolated.

In summary, screening of EC samples with a number of antibodies with potential to mark CSCs produced an array of results. By combining the data obtained from flow cytometry and immunohistochemistry analysis a rational antibody priority list was developed. Further testing of isolated populations based on the expression or absence of the top priority antibody (W6B3) and another closely associated antibody (293-C3), which both react with CD133, will be examined in chapter 4. ECSC identification will allow us to determine if a specific set of EC cells are responsible for the initiation, maintenance and progression of EC in future studies.
### 3.5 Supplementary Tables

**Supplementary Table 3.1 Flow Cytometric Analysis Showing Isolated Type I and Type II Endometrial Carcinoma (EC), and Hyperplasia Cells Reacting with each Antibody from the Panel.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type I EC (n)</th>
<th>SD</th>
<th>Type II EC (n)</th>
<th>SD</th>
<th>Hyperplasia (n)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH3A4A7</td>
<td>0.00</td>
<td>(4)</td>
<td>0.13</td>
<td>(3)</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>4FR6D3</td>
<td>0.15</td>
<td>(3)</td>
<td>7.61</td>
<td>(1)</td>
<td>0.84</td>
<td>(2)</td>
</tr>
<tr>
<td>W7C5</td>
<td>0.58</td>
<td>(4)</td>
<td>0.31</td>
<td>(3)</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>W7C6</td>
<td>0.74</td>
<td>(4)</td>
<td>2.56</td>
<td>(3)</td>
<td>2.2</td>
<td>4</td>
</tr>
<tr>
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<td>1.44</td>
<td>(3)</td>
<td>1.22</td>
<td>(1)</td>
<td>4.88</td>
<td>(2)</td>
</tr>
<tr>
<td>97A6</td>
<td>1.64</td>
<td>(3)</td>
<td>0.01</td>
<td>(1)</td>
<td>7.22</td>
<td>(3)</td>
</tr>
<tr>
<td>DOR5A2B</td>
<td>2.06</td>
<td>(4)</td>
<td>19.31</td>
<td>(2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1/4c4</td>
<td>2.44</td>
<td>(3)</td>
<td>2.67</td>
<td>(3)</td>
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<td>(3)</td>
<td>12.03</td>
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<td>(1)</td>
</tr>
<tr>
<td>48B3</td>
<td>2.82</td>
<td>(4)</td>
<td>10.26</td>
<td>(2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BV10</td>
<td>3.33</td>
<td>(4)</td>
<td>0.87</td>
<td>(3)</td>
<td>1.3</td>
<td>3</td>
</tr>
<tr>
<td>P3C4</td>
<td>5.92</td>
<td>(4)</td>
<td>7.72</td>
<td>(3)</td>
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<td>10</td>
</tr>
<tr>
<td>hNPC</td>
<td>7.05</td>
<td>(3)</td>
<td>-</td>
<td></td>
<td>5.58</td>
<td>(3)</td>
</tr>
<tr>
<td>BrCa</td>
<td>7.94</td>
<td>(3)</td>
<td>7.03</td>
<td>-</td>
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<td>(3)</td>
</tr>
<tr>
<td>2DID12</td>
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<td>(3)</td>
<td>12.43</td>
<td>(1)</td>
<td>3.99</td>
<td>(1)</td>
</tr>
<tr>
<td>W8B2B10</td>
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<td>(2)</td>
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<td>293-C3</td>
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<td>14.31</td>
<td>(3)</td>
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<td>6</td>
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<td>CUB1</td>
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<td>9.86</td>
<td>(1)</td>
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<td>(2)</td>
</tr>
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<td>(2)</td>
<td>-</td>
<td></td>
<td>4.73</td>
<td>(2)</td>
</tr>
<tr>
<td>F9C2C1</td>
<td>15.01</td>
<td>(3)</td>
<td>10.65</td>
<td>(1)</td>
<td>28.93</td>
<td>(2)</td>
</tr>
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<td>67A4</td>
<td>18.49</td>
<td>(3)</td>
<td>13.57</td>
<td>-</td>
<td>4.69</td>
<td>(3)</td>
</tr>
<tr>
<td>W3D5</td>
<td>20.36</td>
<td>(3)</td>
<td>17.98</td>
<td>(4)</td>
<td>16.80</td>
<td></td>
</tr>
<tr>
<td>W6B3</td>
<td>47.44</td>
<td>(3)</td>
<td>7.34</td>
<td>(1)</td>
<td>3.36</td>
<td>(3)</td>
</tr>
<tr>
<td>W5C4</td>
<td>54.28</td>
<td>(4)</td>
<td>48.11</td>
<td>(2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24D2E2</td>
<td>62.21</td>
<td>(3)</td>
<td>-</td>
<td></td>
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<td>(1)</td>
</tr>
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<td>70.36</td>
<td>(3)</td>
<td>58.16</td>
<td>(1)</td>
<td>78.04</td>
<td>(2)</td>
</tr>
</tbody>
</table>

Results are means of (n) samples. SD, standard deviation; n= number of individual EC or hyperplasia samples tested; -, not tested.
**Supplementary Table 3.2 Reactivity of Human Endometrial Carcinoma (EC) to the Antibody Panel.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type I</th>
<th></th>
<th>Type II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strong</td>
<td>medium</td>
<td>weak</td>
<td>strong</td>
</tr>
<tr>
<td>67D2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>293-C3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>W6B3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>67A4</td>
<td>2</td>
<td>2 (m,m)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CUB1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D0R5A2B</td>
<td>1</td>
<td>2 (d,d)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>W3D5</td>
<td>1</td>
<td>2 (d,m)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>51D6</td>
<td>1</td>
<td>1 (m)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>97A6</td>
<td>1</td>
<td>1 (m)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>W8B2B10</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>48B3</td>
<td>0</td>
<td>4 (d,d,m,m)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W5C4</td>
<td>0</td>
<td>4 (d,d,m,m)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>184C3</td>
<td>0</td>
<td>3 (d,d,m)</td>
<td>1</td>
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</tr>
<tr>
<td>1/4C4</td>
<td>0</td>
<td>2 (d,m)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>24D2E2</td>
<td>0</td>
<td>1 (m)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>F9C2C1</td>
<td>0</td>
<td>1 (m)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>W7C6</td>
<td>0</td>
<td>1 (m)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>BV10</td>
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<td>0</td>
<td>0</td>
</tr>
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<td>CH3A4A7</td>
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<td>4</td>
<td>0</td>
</tr>
<tr>
<td>P3C4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>W3C4E11</td>
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<td>0</td>
<td>4</td>
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<td>0</td>
</tr>
<tr>
<td>4FR6D3</td>
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</tr>
<tr>
<td>57D2</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>9A3G9</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

For Type I samples, data presented are the results from 4 samples; for Type II, data present are the result from 1 sample, and are classified based on Table 3.4. Medium intensities are presented in () with d representing darkly stained cells, and m representing medium stained cells.
3.5 Supplementary Figures

67A4

W8B2

CUB1

DOR5A2B

51D6
Supplementary Figure 3.1 Flow Cytometry and Immunohistochemistry Profiles of Remaining Priority List Antibodies. The remaining antibodies from the priority list (in order of priority) may be investigated further if the top two antibodies do not enrich for cancer stem cells in human endometrial carcinoma. Representative flow cytometry (FC, left and middle panels) and immunohistochemistry (inserts left and middle panels) results are shown for Type I (left panels) and Type II (middle panels) samples. Right panels show percentage positive cells on individual endometrial carcinoma samples by FC.
Supplementary Figure 3.2 Flow Cytometry and Immunohistochemistry Profiles of Remaining Antibodies Removed from the Priority List. Antibodies removed from the priority list (in alphabetical order) will not be studied as potential endometrial carcinoma stem cell markers. Representative flow cytometry (FC, left and middle panels) and immunohistochemistry (inserts left and middle panels) results are shown for Type I (left panels) and Type II (middle panels) samples. Right panels show percentage positive cells on individual endometrial carcinoma samples by FC.
Chapter 4: CD133-1 and CD133-2 do not Enrich for Endometrial Cancer Stem Cells
Chapter 4: CD133-1 and CD133-2 do not Enrich for Endometrial Cancer Stem Cells

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4.1 Introduction

CD133 is a five transmembrane glycoprotein homologous to mouse Prominin-1 [250-252]. The CD133 gene is located at 4p15.33 and produces an 865 amino acid protein that is 97kDa in its unglycoslyated form (120kDa glycosylated) [251-254]. While CD133 is often located on protrusions of epithelial cell membranes, its ligand and function is unknown, although it is thought to be involved with cell-cell interactions and mature organ homeostasis [157, 251, 254, 255].

Due to the structure of the gene several splice variants have been hypothesised and two common epitopes of CD133, CD133-1 and CD133-2, are recognised [254-257]. CD133-1, recognised by the antibody AC133 [255, 257, 258], is a glycosylation dependant epitope [255, 257]. CD133-1 was first identified after CD34\textsuperscript{bright} haematopoietic stem cells (HSCs) were used to immunise mice to create monoclonal antibodies against HSCs [253]. Later, the retinoblastoma cell line WERI-Rb-1 was found to express high levels of CD133-1 and was used to immunise mice to generate additional AC133-like antibodies [253]. From these experiments the AC141 antibody (CD133-2) was discovered [255, 257, 259], which recognises a spatially distinct form of CD133 from CD133-1 [253, 255, 257]. It appears that the expression of CD133-1 is more restricted than the expression of CD133-2 [256, 257].

Adult stem cells (ASCs) express a wide variety of cell surface markers. As yet, there is no single marker specific for all human ASCs. CD133 was first identified as a marker for ASCs in the haematopoietic system [250, 254, 260]. Isolated haematopoietic cells with the CD34\textsuperscript{bright}CD133\textsuperscript{+} phenotype possessed many ASC characteristics including; lack of differentiation markers, enrichment for certain haematopoietic colony forming units, \textit{in vivo} self renewal, and ability to reconstitute bone marrow when transplanted into sub-lethally irradiated rats [250, 254, 260, 261]. However, it should be noted that these cells were not able to give rise to all of the haematopoietic lineages [254, 260]. Yet, similar results have been obtained in other human organs (Table 4.1).

In the adult human kidney [262], neural system [263], prostatic epithelium [264], liver [265], and pancreas [266] CD133\textsuperscript{+} cells (± other markers) enriched for cells that function like ASCs. They lacked differentiation markers, gave rise to more differentiated cells (sometimes including other lineages), had high proliferative potential, and engrafted into
animal models recapitulating the original organ (Table 4.1) [253, 254, 257]. Further, upon differentiation the cells often stopped expressing CD133 [254]. These cells also appeared to co-express other stem cells (SCs) markers [254, 266]. For example, prostatic tissue sections investigated by immunohistochemistry revealed that CD133+ prostatic epithelial cells also expressed α2β1 integrins, a marker previously demonstrated to enrich for prostatic SCs [254, 264]. In addition, CD133+ human kidney cells expressed the pluripotency genes often associated with SCs, OCT-4 and BMI-1 [254].

Cancer stem cells (CSCs) have been identified in a number of solid human tumours (section 1.3.2). CD133 has been investigated as a potential CSC marker in a number of these tumours. In human brain tumours, CD133+ (AC133) cells displayed increased proliferation, self renewal, differentiation and tumour initiating potential compared to the CD133− population [168, 234, 253, 254, 267]. Recent studies on colon [158, 164], liver [268, 269], prostate [165], ovarian [160, 270], pancreatic [271] and lung cancers [272], along with melanoma investigations [273] indicated that the CSC population is enriched within the CD133 fraction (Table 4.2) [253, 254, 257]. Cells in the CD133+ fraction demonstrated the CSC properties of long term growth in culture, self renewal, colony forming ability, differentiation and ability to initiate tumours in vivo [254, 257].

While evidence for endometrial cancer stem cells (ECSCs) has been reported (Chapter 2) [230, 231], markers identifying or enriching for these cells are yet to be discovered. In the previous chapter (Chapter 3) a number of candidate markers were identified through a strategic screening process, requiring further investigation to determine if they enrich for ECSCs. Interestingly, this systematic approach identified CD133-1 as the top priority marker to be further examined and CD133-2 as the third. Why other researchers chose to investigate CD133 as a CSC marker for a wide range of tumours (Table 4.2) is uncertain, but the screening approach independently indicates that CD133 may be a potential ECSC marker. For this reason CD133 was investigated as a potential ECSC marker.

The aims of this chapter were to determine if human ECSCs from Type I and Type II tumours were enriched in the CD133 positive subpopulation by investigating the clonogenicity and self renewal potential of the FACS subpopulations. A further sub-aim was to investigate if CD133-1 and CD133-2 co-localised to the same individual EC cells and if this affected the enrichment of ECSCs within the subpopulations.
**Table 4.1 CD133 Expression on Potential Human Stem/Progenitor Cells.**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cell Type</th>
<th>CD133</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>Haematopoietic stem cells</td>
<td>CD133-1</td>
</tr>
<tr>
<td>Periphal blood</td>
<td>Endothelail progenitor cells</td>
<td>CD133-1</td>
</tr>
<tr>
<td>Foetal brain</td>
<td>Neural stem cells</td>
<td>CD133-1/2</td>
</tr>
<tr>
<td>Kidney</td>
<td>Renal progenitor cells, Proximal tubular cells</td>
<td>CD133-1</td>
</tr>
<tr>
<td>Prostate</td>
<td>Basal cells</td>
<td>CD133-1</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatic stem cells, Intraportal ductals, interlobular bile duct cells</td>
<td>CD133-1</td>
</tr>
<tr>
<td>Pancrease</td>
<td>Duct lining cells</td>
<td>CD133-1</td>
</tr>
<tr>
<td>Colon</td>
<td>Epithelial cells at crypt bases</td>
<td>CD133-1</td>
</tr>
</tbody>
</table>

Adapted from Mizrak et al. (2008) and Wu et al. (2009).

**Table 4.2 CD133 Expression on Potential Human Cancer Stem Cells from Solid Tumours.**

<table>
<thead>
<tr>
<th>Cancer</th>
<th>CD133</th>
<th>Cell Source</th>
<th>Type of CSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>CD133-1</td>
<td>Primary tumours, cell lines, primary xenografts</td>
<td>Cancer initiating cells</td>
</tr>
<tr>
<td></td>
<td>CD133-2</td>
<td>Primary tumours</td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>CD133-1</td>
<td>Primary tumours</td>
<td>Subpopulation of CSCs</td>
</tr>
<tr>
<td>Colon</td>
<td>CD133-1</td>
<td>Primary tumours, cell lines</td>
<td>Cancer initiating cells</td>
</tr>
<tr>
<td></td>
<td>CD133-2</td>
<td>Primary tumours</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>CD133-1</td>
<td>Primary tumours</td>
<td>Tumourigenic stem/progenitor cells</td>
</tr>
<tr>
<td></td>
<td>CD133-2</td>
<td>Primary tumours</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular</td>
<td>CD133-1</td>
<td>Cell lines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD133-2</td>
<td>Cell lines</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>CD133-1</td>
<td>Primary tumours, cell line</td>
<td>Cancer initiating cells</td>
</tr>
<tr>
<td>Ovarian</td>
<td>CD133-1</td>
<td>Primary tumours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD133-2</td>
<td>Primary tumours</td>
<td></td>
</tr>
<tr>
<td>Pancreatic</td>
<td>CD133-1</td>
<td>Primary tumours, cell lines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD133-2</td>
<td>Primary tumours</td>
<td></td>
</tr>
<tr>
<td>Renal</td>
<td>CD133-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CSC, cancer stem cell. Adapted from Mizrak et al. (2008) Bidlingmaier et al. (2008), and Wu et al. (2009).
4.2 Methods

4.2.1 Collection of Human Endometrial Carcinoma Samples

Endometrial carcinoma (EC) or hyperplasia samples were collected from 26 women (64 years ± 13.8 years) undergoing hysterectomy. Tumours were graded by histopathologists and comprised 8 grade 1, 2 grade 1-2, 10 grade 2, 3 grade 3 tumours (all Type I), and 3 Type II tumours (Table 4.3). Samples were collected with patients’ written informed consent by the Victorian Cancer BioBank in accordance with ethics approval obtained from Southern Health Human Research Committee C.

4.2.2 Dissociation of Endometrial Cancer Samples to Single Cells

Tissue samples and single cell suspensions were collected and prepared as previously described [101] with minor modifications. Briefly, tissue was manually dissociated into < 0.5 mm fragments and then digested with 4 mg/ml Dispase (Gibco, Invitrogen, Vic, Australia) in Dulbecco’s phosphate buffered saline (DPBS, Gibco), overnight at 4°C with agitation. Samples were filtered through a 40 µM mesh and the backwash from the mesh collected. The filtrate and backwash fractions containing the epithelial cancer cells were further digested in PBS with 1-2 µg/ml collagenase type II (Worthington Biochemical, Freehold, NJ, USA) and 0.64 µg/ml DNase type I (Roche Diagnostics, Mannheim, Germany) for 30 mins, at 37°C with agitation. At this stage the filtrate portion was diluted in bench medium and placed on ice. The backwash fraction was digested for a further 60-120 mins in 5 mls PBS with 1-4 µg/ml collagenase type II, and 0.64-0.128 µg/ml DNase type I. Occasionally 0.2-0.4 µg/ml trypsin (Invitrogen) was used for 15-30 mins to ensure complete dissociation into single cells. Cells from both fractions were combined and filtered through consecutive 40 µM and 35 µM cell strainers, washed in bench medium and pelleted. Following this, cells progressed to sorting or were frozen in dimethyl-sulfoxide (10% in foetal calf serum [FCS, CSL, Melbourne, Victoria, Australia]; Sigma, USA) at -80°C.
Table 4.3 Patient Details.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Type</th>
<th>Grade</th>
<th>Age</th>
<th>Classification</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>I</td>
<td>1</td>
<td>67</td>
<td>EC</td>
<td>MI, A, L</td>
</tr>
<tr>
<td>11</td>
<td>I</td>
<td>1</td>
<td>77</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>I</td>
<td>1</td>
<td>34</td>
<td>EC</td>
<td>A</td>
</tr>
<tr>
<td>44</td>
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<td>51</td>
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<td>A, L</td>
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<td>54</td>
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<td>I</td>
<td>1</td>
<td>87</td>
<td>EC</td>
<td>MI, L</td>
</tr>
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<td>48</td>
<td>I</td>
<td>1</td>
<td>78</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>I</td>
<td>1-2</td>
<td>54</td>
<td>EC, mucinous</td>
<td>A, polyp</td>
</tr>
<tr>
<td>50</td>
<td>I</td>
<td>1-2</td>
<td>58</td>
<td>EC</td>
<td>G2 Ovarain tumour, L</td>
</tr>
<tr>
<td>22</td>
<td>I</td>
<td>2</td>
<td>68</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>I</td>
<td>2</td>
<td>49</td>
<td>EC</td>
<td>L, H</td>
</tr>
<tr>
<td>52</td>
<td>I</td>
<td>2</td>
<td>53</td>
<td>EC</td>
<td>Lyl, MI, H</td>
</tr>
<tr>
<td>53</td>
<td>I</td>
<td>2</td>
<td>58</td>
<td>EC</td>
<td>MI</td>
</tr>
<tr>
<td>54</td>
<td>I</td>
<td>2</td>
<td>61</td>
<td>EC</td>
<td>MI</td>
</tr>
<tr>
<td>55</td>
<td>I</td>
<td>2</td>
<td>62</td>
<td>EC</td>
<td>MI, H</td>
</tr>
<tr>
<td>56</td>
<td>I</td>
<td>2</td>
<td>62</td>
<td>EC</td>
<td>Lyl</td>
</tr>
<tr>
<td>57</td>
<td>I</td>
<td>2</td>
<td>64</td>
<td>EC</td>
<td>MI, H</td>
</tr>
<tr>
<td>58</td>
<td>I</td>
<td>2</td>
<td>77</td>
<td>EC</td>
<td>MI</td>
</tr>
<tr>
<td>59</td>
<td>I</td>
<td>2</td>
<td>83</td>
<td>EC</td>
<td>MI, H</td>
</tr>
<tr>
<td>25</td>
<td>I</td>
<td>3</td>
<td>57</td>
<td>EC</td>
<td>Lyl, MI, foci of SPC &amp; CCC, H</td>
</tr>
<tr>
<td>60</td>
<td>I</td>
<td>3</td>
<td>82</td>
<td>EC</td>
<td>MI, Lyl, H, EN, L, A</td>
</tr>
<tr>
<td>61</td>
<td>I</td>
<td>3</td>
<td>82</td>
<td>EC</td>
<td>MI, Lyl</td>
</tr>
<tr>
<td>62</td>
<td>II</td>
<td></td>
<td>58</td>
<td>Serous papillary</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>II</td>
<td></td>
<td>83</td>
<td>Serous papillary</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>II</td>
<td></td>
<td>71</td>
<td>Malignant Mixed Müllerian Tumour</td>
<td>MI, L, A</td>
</tr>
</tbody>
</table>

A, adenomyosis; EC, endometrial adenocarcinoma; EN, endometriosis; H, Hyperplasia; L, leiomyoma; Lyl, lymphovascular invasion; MI, myometrial invasion; SPC, serous papillary carcinoma; CCC, clear cell carcinoma.

4.2.3 Fluorescent Activated Cell Sorting using CD133

4.2.3.1 Immunolabelling of Endometrial Cancer Cells with either CD133-1 or CD133-2 Antibodies

Freshly isolated or frozen-thawed EC cell suspensions (10^6 - 10^7 cells/ml) were blocked with goat serum before being labelled with anti-CD133-1 (clone W6B3, Dr Hans-Jörg Bühring) or anti-CD133-2 (clone 293-C3, Dr Hans-Jörg Bühring) antibodies or matched
isotype controls (IgG₁, IgG₂b respectively; Table 4.4), for 45 mins at 4°C with agitation. This was followed by labelling with Zenon® Alexa Fluor® 488 conjugated anti-mouse IgG₁ (CD133-1; Zenon® Alexa Fluor® 488 mlG₁ labelling kit; Invitrogen) or Alexa Fluor® 488 conjugated goat anti-mouse IgG₂b (CD133-2; Molecular Probes, USA) for 30 mins. CD133-1 labelled samples were then incubated with blocking reagent (Zenon® blocking reagent; Invitrogen) for 5 mins at room temperature as per manufacturer’s instructions. To remove non reacting antibodies cells were washed three times in PBS and blocked with mouse serum before cells were incubated with R-Phycoerythrin (PE) conjugated anti-CD10 (mouse anti-human, Caltag, Invitrogen), Alexa Fluor® 647 conjugated anti-CD31 (mouse anti-human, Molecular Probes) and allophycocyanin (APC) conjugated anti-CD45 (mouse anti-human; Catlag Laboratories, USA) for 30 mins (Supplementary Table 4.1). After washing cells were then resuspended in FACS buffer (PBS, 2% FCS, 5 mM glucose) containing 7AAD (1 µg/ml, Sigma) and FAC sorted on DAKO Mo-Flo XDP. To produce optimal separation of subpopulations fluorochromes were chosen to minimise overlap of emission spectra (Fig 4.1).

The majority of cells were selected for analysis based on forward vs. side scatter (Fig 4.2A) and dead cells, endothelial cells (CD31+) and leukocytes (CD45+ cells) were electronically excluded from this population (Fig 4.2B). The remaining cells were sorted into 4 subpopulations, based on CD133-1/2 and CD10 (stromal cell marker) expression (Fig 4.2E; Supplementary Figure 4.1), into collection medium (DMEM/F12, 5% FCS, 1% Antibiotics) at 4°C. A minimum of 5,000 events from the isotype controls were used to determine electronic gating (Fig 4.2C, D).

**Table 4.4 Antibodies used for Multicolour FACS.**

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Raised against</th>
<th>Raised in</th>
<th>IgG</th>
<th>Fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD133-1</td>
<td>human</td>
<td>hybridoma</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Anti-CD133-2</td>
<td>human</td>
<td>hybridoma</td>
<td>2b</td>
<td>-</td>
</tr>
<tr>
<td>Anti-CD10</td>
<td>human</td>
<td>mouse</td>
<td>1</td>
<td>PE</td>
</tr>
<tr>
<td>Anti-CD45</td>
<td>human</td>
<td>mouse</td>
<td>1</td>
<td>APC</td>
</tr>
<tr>
<td>Anti-CD31</td>
<td>human</td>
<td>mouse</td>
<td>1</td>
<td>A647</td>
</tr>
<tr>
<td>Anti-IgG1</td>
<td>mouse</td>
<td>-</td>
<td>-</td>
<td>A488</td>
</tr>
<tr>
<td>Anti-IgG2b</td>
<td>mouse</td>
<td>-</td>
<td>-</td>
<td>A488</td>
</tr>
</tbody>
</table>

APC, Allophycocyanin; PE, R-Phycoerythrin; A488, Alexa Fluor 488; A647, Alexa Fluor 647. A647, APC and 7AAD positive events are collected in the same channel.
Figure 4.1 Emission Spectra of Conjugated Antibodies Used for Sorting CD133$^+$ cells from Endometrial Carcinoma Cell Suspensions. While overlapping excitation spectra is preferable (A), where possible, overlapping fluorescent emission spectra was avoided, apart from the negative selection markers (670nm) (B). Conjugated antibodies were excited by two independent lasers, one at 488nm and the other at 633nm (A). This allowed for maximal range of emissions to be collected and analysed on a single FACS (B). Not all commercially available antibodies (primary and secondary) are conjugated to the desired fluorochromes. Fluorochromes used for FACS were CD133-1/2-A488 (green), and CD10-R-PE (blue), which are commonly used together, but their emissions overlap slightly. Band-pass filters remove much of the overlap by collecting spectra within specific bandwidths (530/40, 580/30 and 670/40 nm). However, there is still some overlap and electronic compensation is used to overcome this overlap, allowing the true PE fluorescence intensity to be analysed. The emissions from the antibodies, CD45-APC (black) and CD31-A647 (red), and the 7AAD (purple) emissions were designed to overlap as cells expressing any of these markers were not required for further analysis and thus it is convenient to electronically remove undesired cell populations using a single filter (B).
Figure 4.2 Electronic Gating Strategy for FACS. (A) The side vs. forward scatter histogram, cell debris and erythrocytes are electronically removed or “gated out”. Only cells in R1 were subsequently analysed. (B) Next, only live cells (exclude 7AAD, i.e. 7AAD⁻), and CD45⁻ cells (APC low fluorescence) are kept for further analysis (cells in R2). Then, isotype control gates are electronically determined to exclude < 2% positive cells fluorescence (C, D). Finally, cells are sorted into separate tubes (E).
4.2.3.2 Immunolabelling of Endometrial Cancer Cells for Co-localisation of CD133-1 and CD133-2

Freshly isolated or thawed EC cell suspensions (2-5 x 10⁵ cells/ml) were labelled with anti-CD133-1 and anti-CD133-2 as in section 4.2.3.1. Following this, tubes were incubated with PE conjugated goat anti-mouse IgG₁ (CD133-1; Caltag) and Alexa Fluor 488 conjugated goat anti-mouse IgG₂b (CD133-2; Molecular Probes) for 30 mins. Conjugated anti-CD31, anti-CD45 antibodies and 7AAD were then added to label endothelial cells, leukocytes and dead cells, respectively, and removed as in section 4.2.3.1 (Supplementary Table 4.2). Samples were then analysed for co-expression of CD133-1 and CD133-2.

4.2.4 Clonogenicity of CD133 Endometrial Cancer Cell Fractions

Freshly sorted (4 subpopulations obtained from FACS) and unsorted fractions of single EC cells were cultured at clonal density (300 cells/cm²) as described previously (Chapter 2) [230], except fibronectin (BD Biosciences, North Ryde, Australia) coated tissue culture-ware (BD Biosciences) was used. After 4 weeks in culture, plates were fixed in 10% formaldehyde/PBS for 10 mins and stained with Harris haematoxylin [101]. Clones (> 50 cells) were counted in the 4 subpopulations and averaged when several replicate plates were done, and colony forming efficiency (CE) determined as previously described (Chapter 2) [101, 230].

4.2.5 In vitro Self Renewal of CD133+ Endometrial Cancer Colony Forming Units

Colony forming units (CFUs) from each of the four FACS fractions and unsorted CFUs were subcloned after 4 weeks in culture, as described previously (Chapter 2) [230]. In the epithelial (CD10⁺), viable (7AAD⁻), and unsorted subpopulations, 3 individual primary clones (> 250 cells) with epithelial-like morphology generated by single cells were re-cloned to examine for self renewal. Clones also formed in the sorted stromal subpopulations, CD133⁺CD10⁻ and CD133⁺CD10⁺ fractions, and from these fractions stromal-like clones were subcloned. Well separated individual clones were trypsinised (0.25%) in cloning rings (Sigma-Aldrich) and replated at < 10 cells/cm² to generate
secondary clones [39]. Following another 4 weeks in culture a single secondary clone generated from each primary clone was re-cloned to generate tertiary clones (Fig 4.3). After another 4 weeks in culture plates were fixed and stained as described (Section 4.2.4).

Figure 4.3 Protocol for in vitro Self Renewal of Endometrial Carcinoma CFUs. Isolated endometrial carcinoma cells from any sorted subpopulation or unfractionated cells were cultured at clonal density. After 4 weeks in culture 3 individual clones per subpopulation were re-cloned. This procedure was repeated again if clones formed. Primary and tertiary plates were fixed in 10% formaldehyde and stained with Harris haematoxylin. Remaining clones from secondary plates were collected for RNA extraction.

4.2.6 RNA Isolation and RT-PCR of Sorted CD133 Endometrial Cancer Cell Subpopulations

Self renewal and pluripotency gene expression was assessed in two samples of freshly sorted EC cell fractions (Fig 4.2E) and in unsorted EC cells. RNA was isolated using RNaqueous micro-columns (Ambion) according to manufacturer’s instructions and as previously described (Chapter 2) [230], with minor changes. These changes included, 0.08-0.5 µg of RNA was reverse transcribed to cDNA (Superscript III, random primers,
Invitrogen), and HOXA11, ABCG2, WNT4, and OCT4 (Table 4.5) gene expressions were also investigated.

### Table 4.5 RT-PCR primer details

<table>
<thead>
<tr>
<th>Protein</th>
<th>Direction</th>
<th>Primer</th>
<th>Tm</th>
<th>Cycle no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-CATENIN</td>
<td>FWD</td>
<td>ACAGCACCTTCAGCACTCT</td>
<td>59</td>
<td>35</td>
<td>[309]</td>
</tr>
<tr>
<td></td>
<td>RVS</td>
<td>AAGTTCTGGCTATTACGACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI-1</td>
<td>FWD</td>
<td>ATGTGTGTGCTTTGTGGAG</td>
<td>59</td>
<td>35</td>
<td>[148]</td>
</tr>
<tr>
<td></td>
<td>RVS</td>
<td>AGTGGCTGGTCTTGTGAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX-2</td>
<td>FWD</td>
<td>CCCCCCTGTGGTTACCTCT</td>
<td>64</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RVS</td>
<td>TTCTCCCCCCCTCCAGTTCG</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NANOG</td>
<td>FWD</td>
<td>AAGACAAGGTCCCGGTCAAG</td>
<td>64</td>
<td>40</td>
<td>[148]</td>
</tr>
<tr>
<td></td>
<td>RVS</td>
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</tr>
<tr>
<td>OCT-4</td>
<td>FWD</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>RVS</td>
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<tr>
<td></td>
<td>RVS</td>
<td>GAGGCATTGCTGATGATCTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 4.2.7 Statistical Analysis

Data were analysed using GraphPad Prism software (version 5, San Diego, CA). Gaussian distribution was examined with Kolmogorov-Smirnov normality tests. Mann Whitney U tests were used to compare the number of cells expressing either CD133 epitope and for CE comparisons between two groups. For more than two groups, Kruskal-Wallis tests were performed for multiple CE comparisons. Fishers exact test (more robust test compared to Chi-squared test) was used to compare the self renewal potential between 2 groups. P<0.05 was considered statistically significant.
4.3 Results

4.3.1 Human Endometrial Cancer Samples Contain Cells Expressing CD133-1/2

Screening studies performed in chapter 3 identified CD133-1 and CD133-2 expression in most samples by single colour flow cytometry and immunohistochemistry, and were first and third on the priority list of antibodies. Initial experiments were undertaken to investigate the co-expression of these two epitopes on the same human EC cells. If the two CD133 epitopes were expressed on the same cells in a similar manner (i.e. similar fluorescence intensity) then the two parameter flow cytometry histograms would be expected to have a diagonal distribution (Fig 4.4). From the scatter plots it appears that CD133-2 fluorescence intensity is higher than for CD133-1 on human EC cells (Fig 4.4). The plots also suggest that cells expressing CD133-1 also expressed CD133-2, but that the reverse may not necessarily occur as there is a small population of CD133-2+CD133-1- cells (blue oval Fig 4.4). Further tests were performed to determine if the expression of either CD133 epitope enriched for ECSCs.

To more accurately compare the percentage and type of EC cells expressing either CD133-1 or CD133-2 four colour flow cytometry was used. Erythrocytes, dead cells (7AAD+), leukocytes (CD45+), and endothelial (CD31+) cells were electronically removed (Fig 4.2B). The remaining population was separated into epithelial (CD10+) and stromal (CD10-) cells. CD10 reacts with human endometrial stromal cells in human EC [274, 275] and in other endometrial pathologies including adenomyosis and endometriosis [276, 277].

The percentage of cells expressing either form of CD133 within the epithelial populations was examined. Figure 4.5 and Table 4.6a shows that there was no significant difference in the percentage of cells expressing either CD133-1 or CD133-2. Expression of CD133 in the epithelial compartments of Type I and Type II tumours was similar and were combined for further analysis (Table 4.6b). Similar observations were made for CD133 expression in the stromal compartment for both CD133-1 and 2 epitopes, with no apparent differences between Type I and II tumours. Unfortunately, comparison of CD133 expression between Type I and II EC samples was not possible because only two Type II samples were used in
these experiments (Table 4.6b). These data suggest that there is no significant difference between the expressions of either CD133 epitope in human EC cells.

The majority of examined cells were epithelial (84.69%, median [26.42-96.07%, range]) and both CD133 epitopes were expressed by a smaller proportion of epithelial (CD10^+) cells (7.45% of total epithelial cells, [0-85.75]; Fig 4.5, Table 4.6c). In the stromal fraction (2.85%, [0-19.52] of sorted cells) a smaller fraction were CD133^+ (1.30%, 0-100.00% of total stromal cells) and were detected at a much lower fluorescence intensity compared to the epithelial fraction.

![Figure 4.4 Expression of CD133-1 (W6B3) and CD133-2 (293-C3) on an Individual Endometrial Carcinoma Sample.](image)

*Figure 4.4 Expression of CD133-1 (W6B3) and CD133-2 (293-C3) on an Individual Endometrial Carcinoma Sample.* A representative sample shown (n=6). Black circle, CD133-1^−CD133-2^+ cells, blue oval, CD133-2^+CD133-1^− cells. Green line, expected distribution if a similar number of EC cells expressed both CD133 epitopes in a similar manner.
Figure 4.5 Percentage of Human Endometrial Carcinoma Cells Expressing CD133. Data shown are individual Type I samples sorted with CD133-1 (circles) or CD133-2 (squares) and Type II samples sorted with CD133-1 (normal triangles) or CD133-2 (inverted triangles). Bars are medians. Most CD133 positive cells were restricted to the epithelial population (blue symbols; 5.59% [0-79.57%]), however, most epithelial cells did not express CD133 (black symbols; 74.73% [13.22-96.75%]). A minute proportion of stromal cells expressed CD133 (red symbols; 0.03% [0-2.61%]), but most did not (purple symbols; 2.83% [0-19.23%]).
Table 4.6a Percentage of CD133 Expressing Cells in Endometrial Carcinoma Cell Suspensions.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133+</th>
<th>Combined</th>
<th>CD133-1</th>
<th>CD133-2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>5.59 (0-79.57)</td>
<td>7.86 (0-79.57)</td>
<td>5.66 (0-53.35)</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>CD133-1</td>
<td>74.73 (13.22-96.07)</td>
<td>77.73 (13.22-96.07)</td>
<td>74.73 (14.2-94.07)</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>0.03 (0-2.61)</td>
<td>0.00 (0-2.61)</td>
<td>0.04 (0-1.22)</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>CD133-2</td>
<td>2.83 (0-19.23)</td>
<td>1.71 (0-19.23)</td>
<td>2.83 (0-6.23)</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

Median (range) presented. N=13 for CD133-1 and n=15 CD133-2. Note Type I and II samples are included. P compares CD133-1 and CD133-2 expression. Combined, both CD133-1 and CD133-2 expressions were combined for this column.

Table 4.6b Percentage of CD133 Expressing Cells in each Type of Endometrial Carcinoma Cell Suspensions.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133+</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>5.59 (0-79.57)</td>
<td>2.08, 18.26</td>
<td></td>
</tr>
<tr>
<td>CD133-1</td>
<td>74.73 (13.22-96.07)</td>
<td>70.18, 88.99</td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>0.03 (0-2.61)</td>
<td>0.00, 0.23</td>
<td></td>
</tr>
<tr>
<td>CD133-2</td>
<td>2.83 (0-19.23)</td>
<td>0.31, 1.79</td>
<td></td>
</tr>
</tbody>
</table>

Median (range) presented. N=20 and 2 for Type I and Type II respectively.

Table 4.6c Relative Percentage of CD133 Expressing Cells in each Compartment of Endometrial Carcinoma Cell Suspensions.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133+</th>
<th>CD133-</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>7.45% (0-85.75)</td>
<td>92.55% (14.25-100.00)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Stromal</td>
<td>1.30% (0-100.00)</td>
<td>98.70% (0.00-100.00)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Median (range) presented. N=15. Note Type I and II samples are included. P compares the relative expression of CD133 in each compartment.

4.3.2 Clonogenicity of Human Endometrial Carcinoma Epithelial Cells Sorted for CD133

The clonogenic activity of single EC epithelial (CD10−) and stromal (CD10+) cells was examined to determine whether clonogenic epithelial cancer cells were enriched in the CD133+ fraction. As reported previously [230], the clones initiated by cells in any of these fractions varied in appearance. In the epithelial fractions (from Type I samples) the clones
were mainly composed of cells that resembled normal human endometrial epithelial cells, and occasionally of cells with an epithelial and stromal phenotype (Fig 4.6A, black, yellow and blue sections). In the Type II samples however, clones from all fractions were similar and often consisted of cells that produced spheres and organoid structures attached to stromal-like or epithelial-like adherent clones (Fig 4.6B). The presence of stromal-like cells in the epithelial fractions may represent epithelial-mesenchymal transition, which has previously been reported in Type II samples [194]. Finally, as epithelial CD133\(^+\) and CD133\(^-\) subpopulations from both types of EC were able to initiate clones of daughter cells, this suggests that the clonogenic CSC is not restricted to the CD133 expressing population in either Type I or II EC.

The CE of each subpopulation was not significantly different between the two CD133 epitopes (Table 4.7a) and thus they were combined to determine if any subpopulation was enriched for ECSCs. Firstly, the number of samples with colony forming units/cells (CFUs) in each subpopulation was not significantly different between those sorted on CD133\(^-\)1 or CD133\(^-\)2. In most cases, separate epithelial fractions from the same sample contained CFUs (Figs 4.2, 4.6 and 4.7, Table 4.7a), which produced clones within 7 days of culture. Interestingly, CD133\(^-\)1 expressing epithelial cells from 2/5 samples did not appear to contain CFUs, although the CD133\(^-\)1\(^-\) epithelial population from the same samples were able to initiate clonal growth. The median CE of epithelial fractions based on CD133 expression was similar (P=0.79), probably because of the large variance in CE. Interestingly, the lowest observed CE was obtained from epithelial cells expressing CD133\(^-\) (0.09%, median; [0.01-0.18, range]), though this was not statistically significant. Again, comparisons between Type I and Type II samples were not possible because only two Type II samples were investigated (Table 4.7b), however, the data were similar. Overall this indicates that the ECSC may not be restricted to the CD133 expressing epithelial subpopulation.

Similar results were obtained in the stromal compartment, however, clones derived from the stromal subpopulations (Type I EC) possessed a normal human endometrial stromal phenotype (Fig 4.6A purple and red sections). While there was no significant difference in the CE for stromal cells sorted on either CD133\(^-\)1 or CD133\(^-\)2 (Table 4.7a), only 3/9 CD133 expressing (3/5 CD133\(^-\)1; 0/4CD133\(^-\)2) stromal populations initiated clonal growth, whereas all non-expressing stromal populations contained CFUs. The inability of
CD133+ stromal cells to initiate clonal growth may be an artefact of the low number of these cells. Due to sample size, sometimes only several hundred CD133+ stromal cells were collected. As CFUs are rare, statistically there may not have been sufficient enough cells to contain even one CFU. Despite this, the CE of both stromal fractions was similar (P=0.83). Interestingly, this indicates that CD133 does not enrich for clonogenic cancer associated fibroblasts.
Figure 4.6 Morphology of Human Endometrial Carcinoma Clones in CD133 Sorted Subpopulations. (A) Representative Type I endometrial carcinoma (EC) cloning plates and clonal morphologies are shown. Most clones from the epithelial fractions had morphologies similar to normal human endometrial epithelial clones and EC clones [230]. However, some epithelial fraction derived clones were stromal-like in appearance. Most clones derived from the stromal fractions appeared like normal human endometrial stromal clones [39]. Yellow gates are referred to as “combined” epithelial subpopulation. (B) Representative Type II EC clonal morphologies. Sometimes clones would grow as spheres and organoids attached to stromal-like (left panel) or epithelial-like (left middle panel) clones. Another common clonal morphology was intermediate between epithelial and stromal (right and middle right panels).
Figure 4.7 Cloning Efficiency of CD133/CD10 Human Endometrial Carcinoma Cell Subpopulations. Data shown are means of ≥ 2 replicate plates and were sorted on either CD133-1 or CD133-2. Bars are medians. No significant difference in cloning efficiency was observed between subpopulations (P=0.54). Circles and squares represent individual Type I samples sorted with CD133-1 or CD133-2 respectively. Normal and inverted triangles represent individual Type II samples sorted with CD133-1 or CD133-2 respectively. Combined are both CD133+ and CD133-, CD10- epithelial cells. Viable (7AAD-) cells are from both the epithelial and stromal populations. Unsorted are cells did not go through the FACS.

Table 4.7a Cloning Efficiency of Human Endometrial Carcinoma Cell Subpopulations Sorted for CD133 Expression.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133+</th>
<th>CD133-</th>
<th>Combined</th>
<th>CD133+</th>
<th>CD133-</th>
<th>n</th>
<th>CD133+</th>
<th>CD133-</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>0.09</td>
<td>0.05</td>
<td>0.09</td>
<td>0.09</td>
<td>0.05</td>
<td>4</td>
<td>0.12</td>
<td>0.02</td>
<td>5</td>
<td>0.45</td>
</tr>
<tr>
<td>CD133</td>
<td>0.14</td>
<td>0.38</td>
<td>0.14</td>
<td>0.14</td>
<td>0.38</td>
<td>3</td>
<td>0.00</td>
<td>0.00</td>
<td>5</td>
<td>0.76</td>
</tr>
<tr>
<td>CD133^+/^</td>
<td>0.52</td>
<td>0.52</td>
<td>0.52</td>
<td>0.52</td>
<td>0.52</td>
<td>4</td>
<td>0.24</td>
<td>0.01</td>
<td>5</td>
<td>0.32</td>
</tr>
<tr>
<td>Stromal</td>
<td>0.12</td>
<td>0.15</td>
<td>0.12</td>
<td>0.12</td>
<td>0.15</td>
<td>4</td>
<td>0.09</td>
<td>0.00</td>
<td>3</td>
<td>0.63</td>
</tr>
<tr>
<td>CD133</td>
<td>0.45</td>
<td>0.02</td>
<td>0.45</td>
<td>0.45</td>
<td>0.02</td>
<td>4</td>
<td>2.86</td>
<td>0.72</td>
<td>5</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Data presented are median percentages (range). Combined; both CD133-1 and CD133-2 results were combined. CD133^+/^ and CD10^- epithelial cells. P values are comparisons between the two CD133 epitopes. Note that Type I and II samples are included.
Table 4.7b Cloning Efficiency of both Types of Human Endometrial Carcinoma Cell Subpopulations Sorted for CD133 Expression.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133</th>
<th>Type I</th>
<th>n</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>CD133+</td>
<td>0.15 (0.00-0.59)</td>
<td>7</td>
<td>0.00, 0.09</td>
</tr>
<tr>
<td></td>
<td>CD133-</td>
<td>0.00 (0.00-1.56)</td>
<td>5</td>
<td>0.00, 0.38</td>
</tr>
<tr>
<td></td>
<td>CD133+/−</td>
<td>0.52 (0.00-1.20)</td>
<td>6</td>
<td>0.00, 0.24</td>
</tr>
<tr>
<td>Stromal</td>
<td>CD133+</td>
<td>0.15 (0.00-6.39)</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>CD133-</td>
<td>0.45 (0.00-7.25)</td>
<td>7</td>
<td>0, 3.33</td>
</tr>
</tbody>
</table>

Data presented are median percentages (range). CD133+; CD10− epithelial cells.

4.3.3 Human Endometrial Cancer CD133+ and CD133− Colony Forming Units Undergo Self Renewal In Vitro

Self renewal of the CFUs in each subpopulation was examined by serially subcloning individual CFUs. Epithelial subpopulations (CD133+CD10−, CD133−CD10−, and CD133+/−C10−) subcloned at least once, most at least twice (Tables 4.8a and Supplementary Table 4.2), indicating that single CFUs underwent self renewing divisions to establish new clones. Further, CD133-1 and CD133-2 epithelial sorted cells were equally capable of serially subcloning, as the number of samples able to form clones at each subcloning was not significantly different (P>0.05, Fishers Exact test), thus these data were combined for further analysis. When comparing epithelial CD133+ and CD133− CFUs a similar number of samples were able to undergo secondary and tertiary subclonings (P>0.05, Fishers Exact tests), indicating no enrichment of self renewal potential in the CD133 expressing subpopulation. Additionally, both Type I and Type II EC epithelial CD133 sorted subpopulations had similar self renewal potential in each subpopulation (P>0.05 Fishers Exact Tests, Table 4.8b), even when the two CD133 epitopes were taken into account (Table 4.8a). Similar results were obtained with human EC stromal populations, however, it should be noted that stromal-like clones were subcloned. Together, this demonstrates extensive in vitro self renewal potential of EC CFUs from all subpopulations and suggests that CFUs with increased self renewal potential are not enriched within any CD133 sorted subpopulation in either type of EC, providing further evidence that the ECSC is not enriched in the CD133+ subpopulation in either the epithelial or stromal compartment.
Table 4.8a Proportion of Human Endometrial Carcinoma Samples that Produced Clones.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133(^+)</th>
<th>1(^{\circ}) Clones</th>
<th>2(^{\circ}) Clones</th>
<th>3(^{\circ}) Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>CD133(^+)</td>
<td>8/10</td>
<td>8/10</td>
<td>3/8</td>
</tr>
<tr>
<td></td>
<td>CD133(^-)</td>
<td>7/10</td>
<td>7/9</td>
<td>3/7</td>
</tr>
<tr>
<td></td>
<td>CD133(^+/)-</td>
<td>11/11</td>
<td>10/10</td>
<td>4/8</td>
</tr>
<tr>
<td>Stromal</td>
<td>CD133(^+)</td>
<td>4/8</td>
<td>4/8</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td>CD133(^-)</td>
<td>10/10</td>
<td>9/10</td>
<td>3/8</td>
</tr>
<tr>
<td>Viable (7AAD-)</td>
<td>11/11</td>
<td>9/10</td>
<td>5/8</td>
<td></td>
</tr>
<tr>
<td>Unsorted</td>
<td>7/11</td>
<td>5/11</td>
<td>2/8</td>
<td></td>
</tr>
</tbody>
</table>

Note that there is no significant difference (P>0.05) between the number of samples able to undergo secondary and tertiary self renew divisions for each subpopulation.

Table 4.8b Proportion of Human Endometrial Carcinoma Type I and Type II Samples that Produced Clones.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD133(^+)</th>
<th>1(^{\circ}) Clones</th>
<th>2(^{\circ}) Clones</th>
<th>3(^{\circ}) Clones</th>
<th>1(^{\circ}) Clones</th>
<th>2(^{\circ}) Clones</th>
<th>3(^{\circ}) Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>CD133(^+)</td>
<td>7/8</td>
<td>6/7</td>
<td>2/6</td>
<td>2/3</td>
<td>2/3</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>CD133(^-)</td>
<td>4/7</td>
<td>4/6</td>
<td>1/6</td>
<td>3/3</td>
<td>3/3</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>CD133(^+/)-</td>
<td>8/8</td>
<td>7/7</td>
<td>3/6</td>
<td>3/3</td>
<td>3/3</td>
<td>1/2</td>
</tr>
<tr>
<td>Stromal</td>
<td>CD133(^+)</td>
<td>4/6</td>
<td>3/5</td>
<td>1/4</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>CD133(^-)</td>
<td>7/7</td>
<td>7/7</td>
<td>2/6</td>
<td>3/3</td>
<td>2/3</td>
<td>1/2</td>
</tr>
<tr>
<td>Viable (7AAD-)</td>
<td>8/8</td>
<td>6/7</td>
<td>3/6</td>
<td>3/3</td>
<td>3/3</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>Unsorted</td>
<td>4/8</td>
<td>4/8</td>
<td>0/6</td>
<td>3/3</td>
<td>1/3</td>
<td>0/2</td>
<td></td>
</tr>
</tbody>
</table>

Note that there is no significant difference (P>0.05) between the number of samples able to undergo secondary and tertiary self renew divisions for each type of EC.

4.3.4 Human Endometrial Cancer CD133\(^+\) and CD133\(^-\) Cells Express Pluripotency and Self Renewal Genes

In chapter 2 [230] it was shown that freshly isolated CK8-expressing EC and clonally derived epithelial EC cells expressed *BMI-1*, *β-CATENIN*, *SOX-2*, and *NANOG*. Similarly, the self renewal, pluripotency, and developmental genes *BMI-1*, *β-CATENIN*, *NANOG*, *HOXA11*, and *WNT4* [278-280] were expressed by all Type I EC subpopulations (Fig 4.8), confirming the cancer phenotype and indicating no enrichment of these pathways in the CD133\(^+\) epithelial fraction. *ABCG2* is a potential SC gene marker, producing a product that removes cytotoxic substances from cells, a property of CSCs, and was also found in all epithelial fractions of the Type I tumour. Differences in expression between the epithelial CD133\(^+\) and CD133\(^-\) Type I subpopulations were only observed for *SOX-2* and *OCT4*. In
both cases the CD133− fraction expressed the genes, indicating the CD133+ fraction was not enriched for CSCs. In the epithelial fraction of the Type II sample BMI-1 was not expressed by the CD133-2− fraction, whereas the reverse was observed for β-CATENIN. Interestingly, CD133-2+ but not CD133-2− stromal cells did not express β-CATENIN, suggesting a possible link between CD133 expression and β-CATENIN in Type II EC. A similar link was not observed for BMI-1 in the Type II tumour. Similar results to the Type I epithelial cells were obtained for the pluripotency, developmental, and SC genes NANOG, HOXA11 and ABCG2 in the Type II epithelial fractions, but differences were observed with SOX-2, OCT4, and WNT4. Both epithelial fractions in the Type II sample displayed the same expression, or lack thereof, of these genes, again indicating no enrichment of CSCs in the CD133+ fraction.

Similar results were observed in the stromal fraction of both tumours, although notable differences were observed for WNT4, HOXA11, OCT4, ABCG2, and BMI-1. WNT4 was absent from both stromal compartments from both tumours, indicating that the β-CATENIN-WNT4 pathway is restricted to tumour epithelial cells. Also, HOXA11 was expressed by all stromal compartments except the CD133-2− fraction of the Type I tumour, however the converse occurred with OCT4 expression. This suggests that embryonic pathways are re-activated in the cancer phenotype, though different fractions may activate different genes. In the Type II tumour, BMI-1 and β-CATENIN were only expressed in the CD133-2− compartment, and ABCG2 in neither. This suggests that there is no enrichment of cells with CSC properties in the CD133 expressing portion of the stromal compartment, similar to the epithelial results. The interpretation of these results requires some caution, as only one sample of each EC type was investigated. However, together these data indicate no enrichment of cells with self renewal and pluripotency capacity, potential ECSCs, within the CD133+ fraction of either compartment of both types of EC.
Figure 4.8 Expression of Self Renewal and Pluripotency Genes by Endometrial Carcinoma Subpopulations. RT-PCR of self renewal and pluripotency genes in epithelial and stromal CD133-2 sorted subpopulations from single Type I and Type II endometrial carcinoma samples. Epithelial subpopulations CD133-2/CD10-, CD133-2+/CD10-, CD10+; stromal subpopulations CD133-2/CD10-, CD133-2+/CD10+, CD10+; viable epithelial and stromal cells were 7AAD-; unsorted cell fractions did not pass through the FACS.
4.4 Discussion

These investigations demonstrated that cells with \textit{in vitro} CSC properties were not restricted to the CD133\textsuperscript{+} subpopulation of human EC tissues. While both CD133 epitopes were expressed in EC, CD133\textsuperscript{+} epithelial cells were not enriched for cells with increased clonogenic capacity, self renewal potential, or expression of self renewal, pluripotency, and developmental genes, compared to CD133\textsuperscript{−} epithelial cells for either Type I or Type II tumours.

Many studies have examined the expression of CD133 as a marker of CSCs in human cancers, including, colon [150, 158, 164], ovarian [160, 167], prostate [264], and brain tumours [234, 281], with variable expression being observed (0-70\% of cells). This large variation has been explained by the hypothesis that CSCs are not necessarily rare [139]. Similarly in this study, expression of CD133 in human EC varied considerably from 0-80\% of isolated cells, but was in line with previously reported results (1.3-62.6\%) [247]. Many groups have demonstrated that CD133\textsuperscript{+} cells proliferate more extensively, are more resistant to chemotherapeutic drugs, and are more efficient at forming serially transplantable tumours \textit{in vivo}, compared to CD133\textsuperscript{−} cells [150, 158, 160, 164, 167, 168, 268-270]. My results contradict this finding and indicate that the ECSC is not enriched within the CD133\textsuperscript{+} fraction.

This current study demonstrated that sorted human epithelial EC cells, based on CD133 expression or lack thereof, formed clones at a similar frequency (0.09\%-0.15\%), which was also similar to that previously observed for unsorted EC cells (0.24\%, Chapter 2) [230]. Further, the CD133 epitope did not affect the distribution of CFUs in the sorted subpopulations. In contradiction, Rutella \textit{et al.} found a significantly higher percentage (P=0.001) of CD133-1\textsuperscript{+} cells (14.5\%) were able to initiate clones compared to the CD133-1\textsuperscript{−} fraction (2.1\%) [247]. It is likely that these discrepancies are due to technical differences. Rutella \textit{et al.} cultured single cells in 96 well plates in supplemented MyeloCult medium and counted clones > 30 cells in size [247]. I only counted clones composed of > 50 cells which were cultured at a higher density on plates in supplemented DMEM/F12 media. Rutella \textit{et al.} also demonstrated that CD133\textsuperscript{+} cells proliferated more extensively and were more resistant to chemotherapeutic drugs than CD133\textsuperscript{−} cells, suggesting that ECSC are enriched within the CD133\textsuperscript{+} cell fraction [247]. However, in this study the growth kinetics and changes in CD133 expression of the unsorted populations were not
examined in human EC, and thus it is not known if there is any interaction between the CD133+ and CD133− populations. My study also demonstrated that the CD133 epitope did not affect the ability of CFUs to undergo in vitro self renewing cell divisions and that CD133 expression did not enrich for self renewing CSCs, extending the investigations performed by Rutella et al. [247]. Together, these results are contradictory and require further investigation, especially studies examining in vivo self renewal using serial transplantation methodology.

This study also examined expression of self renewal, developmental, SC, and pluripotency genes within CD133+ and CD133− epithelial sorted fractions from both types of EC. Overall these results were in accordance with previous studies that indicate different molecular pathways are activated in Type I vs. Type II EC [181]. Epithelial subpopulations from both types of EC expressed the self renewal, developmental, and SC genes BMI-1, SOX-2, HOXA11, and NANOG [282], indicating this compartment contains ECSCs in both types of EC. They also expressed ABCG2, a gene associated with removal of cytotoxic substances from a cell, a potential property of CSCs. β-CATENIN is involved in the WNT signalling pathway [182] and is often constitutively activated in Type I EC [76]. In accordance with this β-CATENIN and WNT4 were expressed by both CD133+ and CD133− epithelial cell fractions of Type I EC. Also, the pluripotency gene OCT4 [283] was expressed in the epithelial CD133− subpopulations of the Type I tumour. Together, these results suggest that cells with greater pluripotency capacity and activation of developmental pathways associated with cancer, properties of CSCs, are present in the epithelial CD133− and CD133+ populations of Type I EC. In Type II EC similar results were obtained, although there are several notable differences. Neither β-CATENIN nor WNT4 were expressed in the epithelial subpopulations of the Type II EC, indicating these cells use other pathways for self renewal which may include SOX2, HOXA11 and NANOG. Finally, OCT4 was only expressed in the epithelial subpopulation of Type II EC. These data indicate that ECSCs are not restricted to the CD133+ subpopulation in Type II EC.

Stromal cells were separated in this investigation to ensure that cells with CSC properties were present in the epithelial compartment, where the ECSC is hypothesised to be located. The removal of the stromal cells could explain the differences in CE observed between the Rutella study and the present one. Rutella et al. [247] did not remove EC stromal cells in their investigations. I separated the stromal cells and demonstrated this compartment
contains CFUs that undergo self renewing divisions and expresses self renewal, developmental, and pluripotency genes. While the proportion of stromal cells expressing CD133 was rare, these cells may have contributed to the CE observed by Rutella et al. [247]. Freshly isolated cells were used in the present study rather than cultured cells, since culture can irreversibly changes gene expression patterns of cells [284]. However, Rutella et al. performed a large microarray study (not including the genes I examined) on cultured CD133-1⁺ or CD133-1⁻ derived cells [247]. In my study, the pattern of gene expression in the stromal compartment suggests no enrichment of cells with self renewal and pluripotency capacity, as potential ECSCs were found within the CD133⁺ and CD133⁻ fraction of either compartment of both types of EC. However, extrapolation of these results requires some caution as only single samples of each EC were examined. There is potential to perform future studies to determine if these results are maintained after self renewing cell divisions. It is hypothesised that this would reduce the molecular contamination by progenitor cells which can only undergo limited self renewing divisions.

The different subpopulations that enrich for CSCs within the same tumour may be related to technical issues, particularly associated with the CD133 epitope, because in some organs CD133 epitopes are differentially expressed [285]. Alternatively, CD133 is not the only cell surface marker used to isolate CSCs and in some tumours it may not be useful as a single marker. For example in prostate cancer, α2β1 and CD44 [165, 264], in leukaemia, CD34 [286], and in oligoastrocytoma, nestin [267], have been used in combination with CD133 to isolate CSCs. It is possible that in human EC CD133 is not useful as a marker of ECSCs alone, but may be when combined with another marker. Importantly, not all cancers express CD133 [159, 161-163] and not all CD133⁺ cells have been found to be tumourigenic in other cancers [164, 287]. Other studies have identified tumour initiating cells (TICs) within the CD133⁻ population [287-289]. Also, of interest is that many normal epithelial cells express CD133, including renal tubular, pancreatic and liver ductal cells, salivary gland cells, sweat gland cells [290], and endometrial epithelial cells [291], and not all of these are ASCs.

This was the first work to examine the expression and the in vitro functional CSC properties of human EC cells sorted on either CD133-1 or CD133-2, concurrently. A previous study examined the properties of human EC cells sorted using CD133-1 [247] and demonstrated that most CD133-1 expressing cells also expressed CD133-2 [247]. This
result was also confirmed by this investigation. However, in the Rutella et al. investigation cells were not sorted and functionally investigated using the CD133-2 epitope [247]. As my study found a small population of cells that expressed CD133-2 and not CD133-1, functional investigations on cells sorted individually using both epitopes were performed. Interestingly, my results indicated that the CD133-2 subpopulation includes the CD133-1 population and other cells, however, these additional cells did not have any significant affect on the CSC assays performed. Similar results have been found in ovarian carcinoma, where both epitopes were present on the same cells [160]. Unfortunately, I did not examine enough Type II samples to compare the expression of the CD133 epitopes between the two types of EC, however, the Rutella et al. publication, which only examined CD133-1, did not find a difference in expression between endometrioid (Type I) and non-endometrioid (mainly Type II) tumours [247].

To confirm my findings in vivo, experiments are required that can more accurately recapitulate niche conditions. Rutella et al. subcutaneously transplanted freshly isolated CD133-1⁺ and CD133-1⁻ fractions into NOD/SCID mice, but the cells did not produce tumours [247], however, neither did the unfractionated cells. The inabilities of several unfractionated populations to produce tumours are of concern, and lead me to perform subrenal transplantations of freshly isolated CD133⁺/- human EC cells. Unfortunately these experiments are time consuming and will not be available for analysis for some time and could not be reported in this thesis. Further, Rutella et al. were able to produce tumours of comparable sizes when xenografted tumours derived from primary cells were sorted on CD133 expression and re-transplanted [247], suggesting that the TIC does not necessarily express CD133. Further, more comprehensive analysis of the in vivo properties of CD133 expressing human EC cells is required to determine if this marker enriches for ECSCs.

One limitation of this study was that only two Type II EC and no endometrial hyperplasia samples were investigated. Unfortunately, these samples are relatively rare (approx 1 in 10 samples [76]) and pathological diagnosis is not available at the time of isolation. Future experiments with Type II EC or endometrial hyperplasia derived cell lines or xenografted human EC tumours may help determine if the CD133⁺ subpopulation is enriched for CSCs within these pathologies. Secondly, samples were only separated into subpopulations based on FACS and other techniques for cell sorting, such as magnetic activated cell sorting (MACS), were not investigated. FACS is associated with a high frequency of cell death.
However, the results of the CSC assays in the viable (7AAD− fraction) and unsorted cell populations indicate that FACS does not adversely affect EC cells. Further, separating cells on high/low rather than +/- expression is not possible with MACS. Finally, the in vitro culture conditions may lack niche factors found in the in vivo environment. Likewise, in vivo xenograft assays in mice may not produce growth factors and niche conditions that are suitable for human TICs. Additionally, progenitor cells may behave as SCs in culture, with CFU and self renewal potentials [101, 230]. As a range of clone sizes were observed in epithelial subpopulations, it is possible these factors impacted on the data obtained. Progenitor cells have limited differentiation and self renewal capabilities in vivo [74] and thus subrenal transplantation of the subpopulations into NOD/SCID mice are currently addressing this issue. However, these experiments are long term and require several more months incubation. Nevertheless, the in vitro data indicate that ECSCs are not restricted to the CD133+ subpopulation.

There are a couple of important implications of this work. Broadly, it provides further evidence that CD133+ cells are not enriched for CSCs in all cancers, at least not as the primary marker. Specifically for EC, it suggests that the ECSC is not restricted to the CD133+ population and that other CSC markers need to be investigated, however, more experiments are required to confirm this.

In this study I have demonstrated that cells with in vitro CSC properties of clonogenicty, self renewal and expression of self renewal and pluripotency genes are not enriched within the CD133+ vs. the CD133− subpopulation. While future in vivo investigations are required to confirm that ECSCs are not enriched by sorting with CD133, this study provides evidence that neither CD133-1 nor CD133-2 are useful as markers enabling the isolation of ECSCs. With the continued investigation of CD133 as a marker of ECSC and the future discovery of other markers for ECSCs, it is hoped that ECSCs can be isolated and characterised and that their role in the development of human EC investigated. This knowledge opens the way for the development of new treatment modalities that target the CSC, but spare the remaining normal endometrial stem/progenitor and other cells. Such treatments will be particularly useful for early stage and pre-menopausal EC candidates where the uterus may be conserved, and for late stage cases where hysterectomy is not curative and current treatments target the bulk tumor cells rather than CSC.
### 4.5 Supplementary Tables

#### Supplementary Table 4.1a Setup of CD133-1 Immunolabelling for FACS

<table>
<thead>
<tr>
<th>Tube contents</th>
<th>First incubation</th>
<th>Second Incubation</th>
<th>Third incubation</th>
<th>Fourth incubation</th>
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</thead>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD133/1-488</td>
<td>CD133/1-488</td>
<td>W6B3</td>
<td>Zenon Anti-mouse IgG1-A488</td>
<td>CD10-PE</td>
</tr>
<tr>
<td>CD10-PE</td>
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<td></td>
<td></td>
<td>CD45-APC</td>
</tr>
<tr>
<td>CD31-A647</td>
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<td></td>
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<td>CD31-A647</td>
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<tr>
<td>CD45-APC</td>
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<td>CD45-APC</td>
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<td>CD31-A647</td>
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<tr>
<td>CD45-APC</td>
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<tr>
<td><strong>PE control</strong></td>
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<td></td>
<td></td>
</tr>
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<td>W6B3</td>
<td>Zenon Anti-mouse IgG1-A488</td>
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<td>CD31-A647</td>
</tr>
<tr>
<td>CD45-APC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>AF647/APC control</strong></td>
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<td></td>
</tr>
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<td>Zenon Anti-mouse IgG1-A488</td>
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<tr>
<td><strong>AF488 single colour</strong></td>
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<tr>
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<td></td>
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<td>W6B3</td>
</tr>
<tr>
<td><strong>PE single colour</strong></td>
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<tr>
<td>CD10-PE</td>
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</table>
| Washes in PBS occurred between all incubations. APC, Allophycocyanin; PE, R-Phycocerythrin; A488, Alexa Fluor 488; A647, Alexa Fluor 647. Isotype controls were used to set gates for 2 parameter histograms.
**Supplementary Table 4.1b Setup of CD133-2 Immunolabelling for FACS**

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<td>Goat anti-mouse IgG2b-488</td>
<td>CD10-PE CD45-APC CD31-A647</td>
</tr>
<tr>
<td></td>
<td>CD10-PE CD31-A647</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD45-APC</td>
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</tr>
<tr>
<td>AF488 control</td>
<td>mlgG2b-488</td>
<td>mlgG2b</td>
<td>Goat anti-mouse IgG2b-488</td>
<td>CD10-PE CD45-APC CD31-A647</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD45-APC</td>
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</tr>
<tr>
<td>PE control</td>
<td>CD133/1-488</td>
<td>293-C3</td>
<td>Goat anti-mouse IgG2b-488</td>
<td>mlgG1-PE CD45-APC CD31-A647</td>
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<td>AF647/APC control</td>
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<td>PE single colour</td>
<td>CD10-PE</td>
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<td>CD10</td>
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</table>

Washes in PBS occurred between all incubations. APC, Allophycocyanin; PE, R-Phycoerythrin; A488, Alexa Fluor 488; A647, Alexa Fluor 647. Isotype controls were used to set gates for 2 parameter histograms.

**Supplementary Table 4.2 Setup of CD133-1 and CD133-2 Immunolabelling for flow cytometry**

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<th>Third Incubation</th>
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<td>W6B3</td>
<td>Goat anti-mouse IgG1-PE</td>
<td>CD45-APC</td>
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<td>CD133/2-488</td>
<td>293-C3</td>
<td>Goat anti-mouse IgG2b-488</td>
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Washes in PBS occurred between all incubations. APC, Allophycocyanin; PE, R-Phycoerythrin; A488, Alexa Fluor 488; A647, Alexa Fluor 647.
<table>
<thead>
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<th>Subpopulation</th>
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<th>3° Clones</th>
<th>1° Clones</th>
<th>2° Clones</th>
<th>3° Clones</th>
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<tbody>
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<td>3/5</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td>CD133⁻</td>
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<td>4/4</td>
<td>1/2</td>
<td>3/5</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td>CD133⁺⁻⁻</td>
<td>6/6</td>
<td>5/5</td>
<td>1/3</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Stromal</td>
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<td>0/1</td>
<td>2/4</td>
<td>2/4</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>CD133⁻</td>
<td>5/5</td>
<td>5/5</td>
<td>1/3</td>
<td>5/5</td>
<td>4/5</td>
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</tr>
<tr>
<td>Viable (7AAD⁻)</td>
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</tr>
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</table>
Supplementary Figure 4.1 Histogram Before and After Compensation. From figure 4.1 it can be seen that the AF488 emission spectra overlaps into the PE emission spectra. To allow the “true” emission of a cell to be determined, compensation is required. The emission spectra before (A) and after (B) compensation are shown.
Chapter 5: Discussion and Future Directions
Chapter 5: Discussion and Future Directions

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5.2.1 CD133 as a Marker of Endometrial Cancer Stem Cells ................................. 132

5.3 Limitations of this Investigation 135

5.4 Implications and Future Directions 137
This study was designed to test the hypothesis that human endometrial carcinoma (EC) arises from cancer stem cells (CSCs), a rare, phenotypically distinct subpopulation of cells within the cancer. Further, these CSCs could be isolated on the basis of selective marker expression and in particular, are enriched within the epithelial CD133 expressing subpopulation of EC cells, a potential CSC marker identified through a strategic screening process.

When this investigation was begun the only evidence for CSCs in human EC was based on a carcinosarcoma cell line [229]. This evidence was limited, as the study demonstrated colony forming units (CFUs) were present within cells from a carcinosarcoma derived cell line [229]. Therefore, evidence for the existence of CSCs in fresh human EC tissues was lacking. Initial studies (Chapter 2) [230] revealed that a small number of freshly isolated human EC cells from both types of EC possessed the CSC properties of clonogenicity, tumour initiation, differentiation, and self renewal [230]. However, it was not known if a single subtype of human EC cells was capable of all of these properties (potential endometrial cancer stem cells [ECSCs]), or if the properties were distributed across several subtypes of phenotypically distinct cells. To determine if specific cell surface markers could prospectively isolate potential ECSCs a range of cell surface marker expression profiles on human EC cells were examined by flow cytometry (FC) and immunohistochemistry (IHC; Chapter 3). Based on this screening a priority list of candidate ECSC markers was developed, which identified CD133 as the top priority marker. Extensive in vitro CSC assays of human EC epithelial cell subpopulations, sorted on the basis of CD133 expression, were performed (Chapter 4). This study indicated that CD133 expression does not enrich for ECSCs with clonogenic and self renewal properties in either type of EC. Further investigations using other listed priority antibodies may identify a potential marker, or combination of markers that allow the prospective isolation of ECSCs. Once such marker(s) are identified then the ECSC phenotype and function in the initiation, maintenance and development of EC can be studied.
5.1 Identification of Cancer Stem Cell Activity in Human Endometrial Cancer Samples

The first aim of this study was to determine if a subset of cells with CSC properties was present in human EC tissues, and then to estimate their frequency. If such a subpopulation of human EC cells exhibit typical CSC properties of proliferation, tumourigenicity, differentiation, and self renewal, then the CSC hypothesis would be proven for EC as it indicates there is a hierarchy of EC cells.

At the commencement of these studies only a few investigations into CSCs in solid human tumours had been reported in breast, brain, and prostate cancers [165, 168, 169, 234], and these studies mainly focused on higher grade and metastatic tumours [165, 168, 169, 234]. This study was among the first to show that low grade, common human tumours contained a small population of cells with CSC properties [230]. Functional assays designed for investigating normal human endometrial epithelial stem/progenitor cells were adapted to study primary EC and endometrial hyperplasia tissues (Chapter 2) [230]. These assays revealed that a small population of freshly isolated EC and hyperplasia cells exhibited characteristics of CSCs allowing them to proliferate, self renew and generate additional CSCs, as well as differentiate into diverse tumour cells in vivo [230]. These cells occurred at a similar frequency as observed in other human solid tumours [145, 149, 158, 164, 169, 231, 234, 245, 292-294] and normal human endometrial epithelial cells [101, 230]. Interestingly, a range of cell clone and xenografted tumour sizes was observed [230], indicating a hierarchy of cells within EC, suggesting that some have progenitor characteristics. Importantly, this project discovered that not all cells within EC possessed the same clonogenic, tumour initiating, and self renewal properties, conforming to the CSC hypothesis and was the first to demonstrate that this occurred in all grades of Type I EC, Type II EC and its precursor lesion, endometrial hyperplasia [230]. These data suggest that a subpopulation of EC cells are CSCs with similar properties to CSCs characterised in mammary, colon, pancreatic, and prostate cancers, producing further evidence that CSCs play a role in the initiation and progression of malignant human tumours. However, these studies were retrospective and a cell surface marker or combination of markers for the isolation of ECSCs is required so that they can be isolated, characterised and their role in the development of the disease studied.
5.2 Markers Identifying Endometrial Cancer Stem Cells

In 2007 the only markers known to enrich for human CSCs were CD44, CD133, and α₂β₁ identified for breast, brain and prostate cancers [151, 165, 168, 169, 234]. To identify potential ECSC markers (Chapter 3) we took advantage of the heterogeneity of EC cells (identified in chapter 2) [230], hypothesising that ECSCs would express a different combination of markers from their progenitor and more differentiated daughter cells. A panel of potential adult stem cell (ASC) antibody supernatants with specificity for known and novel antigens was investigated. This was the first investigation to identify the expression of the cell surface markers on EC using this panel, and for many of the antibodies on any cancer. Additionally, this work may be extended to identify markers present on more aggressive cancers and hyperplasias susceptible to malignant transformation. Ideally, a potential ECSC antibody marker would react strongly with a small number of EC epithelial cells. However, this was not observed and the antibodies were arranged into a priority list based on other qualities identified in the flow cytometry (FC) and immunohistochemical (IHC) screening of both types of EC and endometrial hyperplasia samples. These included the percentage of cells expressing the marker, the marker’s robustness and consistency of expression between samples. Based on this priority list, 2 antibodies were tested using the functional assays previously developed (Chapter 2) [230] to indicate if they selected for ECSCs in both types of EC.

5.2.1 CD133 as a Marker of Endometrial Cancer Stem Cells

Based on the antibody priority list developed through a rational screening process in chapter 3, the CD133-1 antibody (W6B3) was identified as the top potential ECSC marker in our quest to find the first set of markers that could define ECSCs. Interestingly, the number 3 ranked antibody was the CD133-2 antibody (293-C3). At this time, reports were appearing that indicated there were differences in expression of these two CD133 epitopes [160, 253, 254, 256, 257], and this was the first study to concurrently investigate the expression of the two CD133 antibodies in EC cells to determine if either or both were potential ECSC markers.
It was discovered that neither antibody enriched for epithelial EC cells with in vitro CSC properties, similar to results found in human ovarian cancer [167]. Both CD133 antibody epitopes reacted with a similar proportion of cells in the two types of EC, however, it appears that the larger CD133-2^+ subpopulation includes the CD133-1^+ population, in addition to other cells. Similar percentages of clonogenic cells (0-0.5%) were observed in the unfractionated and all four CD133-1 or CD133-2 sorted populations, indicating no enrichment of CFUs. Further, CFUs from each subpopulation were equally able to undergo self renewing divisions in vitro, with no observable difference between CD133-1 and CD133-2. Finally, expression of self renewal and pluripotency genes (NANOG, β-CATENIN, WNT4, OCT4, BMI-1, SOX2, ABCG2 and HOXa11) in a single sample of both types of EC indicated that the CD133^+ subpopulation was not enriched for ECSCs as the expression of these genes was similar between the epithelial sorted CD133 fractions.

During the course of this study a similar investigation was published with contradictory findings [247]. Rutella et al. discovered that CD133-1^+ cells had a greater cloning efficiency, proliferation rate, cytotoxic drug resistance, oestrogen responsive proliferation and expression of oestrogen receptors, than the CD133-1^− population [247]. However, stromal cells were not removed from their investigations, which may account for the discrepancy as our investigation demonstrated that rare stromal EC cells expressed CD133 and could form clones that underwent self renewing divisions. In contradiction to their own in vitro findings, Rutella et al. found that subcutaneously transplanted freshly isolated CD133-1^+ or CD133-1^− cells were incapable of forming tumours in vivo [247], however, tumours also failed to develop from transplants of unsorted cells from the same samples. This suggests that these samples were devoid of tumour initiating cells (TICs) and it is therefore difficult to draw conclusions. Interestingly, CD133-1^+ and CD133-1^− fractions isolated from primary xenografts derived from unsorted cells formed tumours of comparable sizes when re-transplanted in NOD/SCID mice. This indicates that TICs may be present in both the CD133^+ and CD133^− fractions, especially after the cells have had the opportunity to adapt to the mouse kidney microenvironment [247], and compliments the in vitro discoveries found during my investigation. Together, these data highlight the importance of undertaking a range of in vitro and in vivo CSC assays to confirm the utility of potential markers to enrich for CSCs. While Rutella et al. only investigated the properties of CD133-1 sorted EC cells [247], my results suggest that no difference would
be found between CD133-1 and CD133-2 sorted EC cells. These studies also indicate that more research is required to confirm if CD133 or its epitopes are markers for ECSCs.

Several other cell surface markers, including CD44, CD24, and THY1(CD90) also appear to be common to several cancers [139] and are being investigated in our laboratory as potential ECSC markers. However, further investigation of these markers is required as some tumours have only been investigated by a single laboratory, different laboratories have found different markers for the same tumour types, and a potential CSC marker is not always expressed in every individual case of the cancer [139, 155]. The exact function of these markers and CD133 is yet to be determined, and they may have roles in essential stem cell functions, although this is unlikely given that they are also present on more differentiated cells [139]. Other markers to intracellular targets have been useful in confirming CSC activity of a subpopulation of cancer cells. An example is *MUSASHI-1*, which appears to mark ASC in the intestine and neural system [156], and progenitors of the gastric mucosa, intestine, mammary glands, epidermis and hair follicles [108, 295]. It may be useful as a confirmatory marker in subpopulations of cells obtained using specific cell surface markers. *MUSASHI-1* expressing single cells were recently found in sections of EC samples [108]. It was hypothesised that these were EC progenitor or CSCs. However, functional assays of CSC activity [74] are required to confirm that *MUSASHI-1* identifies CSC in human EC. This marker should be examined in the CD133$^+$ and CD133$^-$ subpopulations. Similarly, *MUSASHI-1* should be examined in phenotypically distinct subpopulations that enrich for ECSCs, once they have been identified.
5.3 Limitations of this Investigation

Limitations of this study mainly concern the consistent availability of human EC samples. These issues are common when using fresh human tissue and also occurred in this study. Firstly, there was the irregularity of sample availability which averaged a sample every 2-3 weeks and there were weeks when no samples were received. This was compounded by the need to process samples quickly and hindered by access to core facilities at short notice (FACS, animals and appropriate biohazard hoods for performing the experiments), lack of diagnosis at the time of sample processing, and small sample size. The number of cells obtained from a sample varied and often limited the number and type of experiments that could be performed. Potentially for similar reasons, most other CSC studies have investigated only 5-15 individual samples [148, 161, 165, 169, 234]. Strategies to overcome these limitations, such as freeze-thawing of isolated cells, were not useful as cryopreservation of EC cells affected functional studies due to cell death. Further, preliminary examinations indicated that certain cell populations did not survive the freeze-thaw process, as the expression of a number of cell surface markers changed. Unfortunately too late for this study, these problems may be overcome by passaging EC cells in immunocompromised mice to generate a stock of in vivo passed EC cells, potentially overcoming the limitations of cryopreservation, culturing, unknown pathological diagnosis, availability, and small sample size [296]. Alternatively, concurrent studies on primary tissues, in vivo passed tumours, cell lines and animal models, would be ideal for future research into this cancer.

Although FACS of single cell suspensions is the optimal approach for sorting subpopulations of cells based on marker expression, it is not without limitations. Enzymatic isolation of single cells from human EC tissues results in cell attrition, however, there is currently no other method for isolating single cells in this and many other tissues. Other methods for sorting cells are available, including magnetic bead activated cell sorting (MACS), however, they rely on single marker expression and cannot distinguish between high/low expressions, which may be required in the future. Also, it does not appear that FACS adversely affects human EC cells as the cloning efficiency and self renewal potential of in vitro cultured cells before and after FACS were similar (Chapter 4, viable vs. unsorted fractions). Further, the culture conditions need to be considered. Serum media may cause differentiation of cells [297, 298] and was used in this study as there was
no information regarding culturing freshly isolated human EC cells for CSC analysis. Various commercial and “home made” serum free-media culture conditions were tested at the beginning of these studies, however, a significant reduction in cloning efficiencies and smaller clonal sizes was observed. Recently, many commercially available serum-free media have been released and are currently being investigated. Non-adherent sphere cultures were not used to determine cloning efficiency as it cannot be determined if individual spheres arose from a single cell or if multiple spheres co-aggregated [299, 300]. Single cell per well deposition was not used as the size of clones could not be accurately determined [39]. Further, the low frequency of CFUs would have made this technique unmanageable. Limiting dilution culture conditions were performed to overcome this problem, but were too variable to fit statistical models. Finally, xenografts were placed subrenally, rather than in an orthotopic site. Orthotopic sites provide more appropriate niches [139], but unfortunately cells are expelled from the uterus before they attach. The renal capsule appears to be the best heterotopic site [301], due to the good blood supply. However, this ability of EC cells to adapt to new environments may be considered as another CSC property. Despite these limitations my PhD studies have still demonstrated heterogeneity in EC cell function, with a small percentage of cells behaving as ECSCs, and have indicated that these cells are not restricted to the CD133 expressing population.
5.4 Implications and Future Directions

The implications of this study relate to other investigations in the CSC field and specifically to EC. In relation to the CSC field, this work adds to the body of evidence that solid human tumours contain CSCs that initiate, maintain, and promote the development of the tumour. Specifically, this study provided further evidence for CSCs in lower grade, early stage, common tumours, an idea that emerged during my candidature [231]. Secondly, a range of potential stem cell markers have been identified, which may be useful in identifying CSCs in other epithelial derived carcinomas. Finally, during my candidature CD133 emerged as a potential cell surface marker that could be used to isolate CSCs in all organs [139, 255, 257]. Recently, studies have been published demonstrating not all CSCs express CD133 [164, 287-289]. However, caution is required when interpreting these results. Several potential promoters have been identified in the CD133 gene [255] and differential expression of the two common CD133 epitopes occurs between [255, 257] and perhaps within organs. In studies where CD133 was shown to enrich for CSCs generally the AC133 (CD133-1) epitope was examined. It is possible that this epitope alone is more useful in identifying CSCs in solid human tumours. However, based on the results from this study, this may not apply to EC.

There are some implications from this investigation that specifically relate to EC. Importantly, these investigations were performed on whole EC samples within 24 hours of surgery. This produces results that are more likely to mimic the original tumour, as compared to EC cell lines. The antibody priority list provides several potential ECSC markers that await investigation as it is unlikely that CD133 enriches for EC TICs. Some of these antibodies are novel and have not been investigated on cancerous tissues. Eventually, separate antibodies may enrich for ECSCs for each type of EC, and perhaps even the subtypes of Type II EC. It is also possible that the cell surface marker for Type I ECs is similar to that found in endometrial hyperplasia, its precursor lesion. Alternatively, an extensive immunohistochemical examination of more EC and hyperplasia samples using these antibodies may identify hyperplasia samples susceptible to malignant transformation, as indicated by genetic screens [302], or in identifying ECs more likely to metastasise. Further, this work implies that CD133 is not useful as a primary marker to isolate cells with in vitro CSC properties in EC. In the future, further in vivo studies will be required to confirm that CD133 is/not a marker of ECSCs. In chapter 2 [230] unsorted cells
were transplanted subrenally and it is possible that stromal cells or cancer associated fibroblasts promoted the growth of the TIC. When CD10 depleted subpopulations are transplanted stromal co-transplantation may be required to provide the appropriate niche, as was observed for human mammary epithelial cells [35], however, Rutella et al. did not remove stromal cells and xenotransplants still failed to grow [247]. Further, it will be important to determine if TIC frequency alters when more severe immunocompromised mice than NOD/SCID mice (NSG mice) are used for transplantation, as has been previously observed [139]. It will also be important to determine if other antibodies on the priority list are useful in isolating ECSCs, so that their involvement in the establishment, progression and maintenance of these tumours can be discovered.

Another important consideration is that it is hypothesised that a single marker will not be sufficient for isolating ECSCs, as has been demonstrated in other tumours [144, 157, 163, 165, 169, 245, 303]. Further, histological classification and staging is created based on patient outcomes and not for medical research. CSC markers may not be consistent between similarly staged and graded tumours. This may become relevant when considering that ECs of the same grades and stages display different molecular backgrounds [76], and it is possible that the generation of CSCs within these tumours results in differential expression of cell surface markers [139]. This will become more apparent when markers isolating ECSCs are found. With the isolation of ECSCs, their genetic profile can be determined and compared with remaining EC cells’ genetic profile. It is hypothesised that this will allow early molecular lesions that result in carcinogenous to be identified.

In cancers with strong evidence for CSCs, including EC, a new cellular target is available for drug development that focuses on eradicating the CSC to prevent tumour recurrence. At present, no such treatments for solid human tumours exist. Combining these future drugs with traditional cytotoxic agents targeting the remaining cancer cells [178] will be essential to ensure that tumour cells do not mutate and acquire self renewal properties and become CSCs themselves. Similarly, ensuring terminal differentiation of all remaining tumour cells, such as differentiation-inducing all-trans retinoic acid (ATRA) for the treatment of acute promyelocytic leukaemia [304], to prevent re-acquisition of CSC properties will be important. Targeting the CSC may be especially important in higher grade and advanced tumours where evidence indicates that the frequency of CSCs is greater than in lower grade and lower staged tumours [139, 141], and also in tumours where the majority of tumour
cells have already been eradicated [178]. It will be important that new treatment options targeting CSCs spare normal stem cells, or are delivered in a targeted manner that avoids normal stem cell exposure.

In this study I have demonstrated that rare cells within a large number of samples of all grades and types of EC are capable of forming colonies, have TIC and differentiation capacity, and can self renew. I have demonstrated that EC cells are heterogeneous in their expression of cell surface markers and have used this property to develop an antibody priority list to identify potential defining ECSC markers. Further, I have examined the in vitro CSC properties of CD133-1 or CD133-2 and CD10 sorted EC cells, and evidence suggests that separation of CD133-1 or CD133-2 expressing EC cells does not enrich for ECSCs. This study lays the groundwork for future studies to identify further markers enabling the prospective isolation of ECSCs required to confirm their existence and role in the development of human EC. Together, with the identification of normal human endometrial epithelial stem cell markers the development and progression of EC can then be investigated, allowing the identification of potential drug targets selective for CSCs, but sparing normal endometrial stem cells. Such treatments will be particularly useful for early stage EC and hyperplasia candidates where the uterus may be conserved, and for late stage cases where hysterectomy is not curative and current treatments target the bulk tumour cells rather than CSC.
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A cancer stem cell origin for human endometrial carcinoma?

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Abstract

Endometrial cancer (EC) is the most common gynaecological malignancy affecting women in the western world. Cancer stem cells (CSCs) are defined as a subset of tumour cells with the capacity to self-renew and give rise to the differentiated cells that comprise the bulk of the tumour. Given that a rare population of epithelial stem/progenitor cells has been identified in human endometrium, it is possible that these cells or their progeny may be the source of the putative CSCs that may initiate and maintain EC. Studies have shown that some cells within EC have the capacity to initiate clones that undergo self-renewing cell division and form tumours in vivo that can be serially passaged, demonstrating self-renewal, proliferation and differentiation abilities of the potential EC stem cells (ECSCs). These potential ECSCs may be located within the tumour cell population expressing CD133 and/or within the side population. With the discovery of markers for ECSCs, it is hoped that ECSCs can be isolated and characterised, and that their role in the development of human EC was further investigated. This knowledge opens the way for the development of new treatment modalities that target the CSCs, but spares normal endometrial stem/progenitor cells and other cells. Such treatments will be particularly useful for early-stage and pre-menopausal EC candidates where the uterus may be conserved, and for late-stage cases where hysterectomy is not curative and current treatments target the bulk tumour cells rather than CSCs.

Reproduction (2010) 139 1–11

The cancer stem cell concept

Many human cancers are composed of cell populations with heterogeneous immunological and genetic profiles, proliferation potentials and differentiation capacities (Heppner 1984, Visvader & Lindeman 2008). To accommodate this heterogeneity, the cancer stem cell (CSC) hypothesis has been proposed. CSCs are defined as a subset of tumour cells with the capacity to self-renew and give rise to the differentiated cells that comprise the bulk of the tumour (Reya et al. 2001, Visvader & Lindeman 2008, Rosen & Jordan 2009). It also suggests that tumours are similar to an organ where there is a hierarchy of cells with varying differentiation and proliferation capacities.

Differences in proliferation potential of the component cell types have been observed in numerous cancers, where it has been demonstrated that only a few cells from the cancer form clones in vitro (Hamburger & Salmon 1977, Reya et al. 2001, Bapat et al. 2005, Tokar et al. 2005, Zhang et al. 2008), and autotransplantation of malignant cells in humans did not always establish a tumour (Southam & Brunschwig 1961, Visvader & Lindeman 2008). Furthermore, several studies have demonstrated that not all human cancer cells xenografted into immunocompromised animal models were able to initiate tumours (Lapidot et al. 1994, Patrawala et al. 2006, 2007, Vermeulen et al. 2008, Zhang et al. 2008). This indicates that only certain cells within the tumour retain the capacity to initiate, maintain and promote the development of the tumours, supporting the CSC hypothesis.

Evidence for the CSC theory has been demonstrated in leukaemic cells, where the CSCs represented <1% of the total cell population (Lapidot et al. 1994, Bonnet & Dick 1997, Rosen & Jordan 2009). It was hypothesised that CSCs expressed cell surface markers or a combination of specific markers that distinguish them from the bulk of the tumour populations, and these can be used to purify populations of CSCs in the same way that normal adult stem cells (ASCs) can be isolated from the remainder of the organ in which they reside. The CSC theory, although controversial, has important implications for our understanding of the biology and development of cancers and offers an alternative target for anti-tumour therapies.

Properties of cancer stem cells

By definition, CSCs have similar properties to ASCs. CSCs may not necessarily acquire the first genetic mutation that initiates tumourigenesis, but they are the cells that maintain the tumour over time
(Visvader & Lindeman 2008). Subsequent mutations within a cancer may create new CSCs that overtake or coexist with the older CSCs (Visvader & Lindeman 2008). Whatever the mutation, the CSCs must retain the capacity to self-renew. To date, the best in vivo indicator of CSC self-renewal is the serial transplantation of CSC-enriched populations into immunocompromised mice, where the tumour is re-established, with a similar phenotype to the original tumour with each serial xenografting (Visvader & Lindeman 2008). This technique also demonstrates that CSCs are able to give rise to large numbers of differentiated progeny. CSCs may also be relatively quiescent, allowing them to escape the cytotoxic effects of chemotherapeutic agents targeting rapidly cycling cells. Chemotherapeutic failure can also be acquired by upregulating drug transporters (Visvader & Lindeman 2008, Rosen & Jordan 2009), which rapidly transport harmful agents from the cell before they exert their effects. Emerging evidence also indicates that CSCs may be less radioreistant than the majority of cancer cells (Diehn et al. 2009). It is hypothesised that CSCs possess DNA protective mechanisms that prevent the effects of radiation (Diehn et al. 2009).

Recent evidence indicates that CSCs may not be as rare as originally thought (Visvader & Lindeman 2008, Rosen & Jordan 2009). In normal organs, homeostatic mechanisms tightly regulate the proliferation of the normal ASCs, maintaining their numbers at relatively low levels. In cancer, these mechanisms no longer apply and CSCs may self-replicate to expand the mutated stem cell pool (Rosen & Jordan 2009). However, numbers of CSCs are often inferred from the percentage of cells expressing a particular surface marker phenotype, which may also be present on more differentiated cells. CSCs and ASCs have many similar properties which may operate through similar molecular pathways, albeit aberrantly in CSCs.

Source of cancer stem cells

There appear to be several sources from which CSCs can arise. They may arise from normal ASCs, from more restricted progenitor cells or even from differentiated cells (Reya et al. 2001, Visvader & Lindeman 2008). Normal stem cells are the likely targets of mutagenesis leading to the formation of CSCs as they already possess active self-renewal pathways, whereas induction of self-renewal genes is required to transform differentiated cells. Furthermore, normal stem cells are the only cells with a lifespan long enough to accumulate all the genetic mutations that lead to tumourigenesis (Reya et al. 2001). To date, CSCs and their ASC counterparts have similar surface marker phenotypes (Lapidot et al. 1994), lacking differentiation markers, but it is unclear whether these markers are related to stem cell functions. Finally, some CSCs have been located in the same region as their normal ASC counterparts, and it is possible that niche signals attract the CSCs to the niche rather than the CSCs arising from the normal ASC (Rosen & Jordan 2009). The ASC niche is composed of one or more cells that protect and maintain the ASCs in a quiescent, undifferentiated state. Niche cells sense the environment and signal to ASCs to divide when new tissue cells are required (Fuchs et al. 2004). It is possible for progenitors and other differentiated cells to give rise to CSCs, although they would have to acquire more genetic mutations, particularly in self-renewal genes (Visvader & Lindeman 2008, Rosen & Jordan 2009). Similarly, it has not yet been determined whether a mutant niche can cause a tumour. It is possible that niche cells receive the first genetic mutations for tumourigenesis, sending aberrant signals to the resident ASC, conferring CSC properties. It has been hypothesised that CSCs arising from normal stem cells result in more aggressive cancer phenotypes, whereas those arising from progenitor cells are less aggressive, though this remains to be proven (Visvader & Lindeman 2008).

Cancer stem cell markers

Cells express a variety of markers on their cell surface. The expression or absence of these markers has been used to isolate subpopulations of cancer cells for the examination of CSC properties. Some cell surface markers including CD133 (PROM1), CD44, CD24 and THY1 (Table 1) are common to several cancers (Visvader & Lindeman 2008). Investigations into solid tumour CSC markers require further investigation as markers in some tumours have only been investigated by a single laboratory, different laboratories have found different markers for the same tumour types, and a potential CSC marker is not always expressed in every tissue.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Additional markers</th>
<th>Tumour type</th>
<th>References</th>
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<tbody>
<tr>
<td>CD44</td>
<td>Head and neck, Breast</td>
<td>Prince et al. (2007), Al-Hajj et al. (2003)</td>
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<td>CD24</td>
<td>Colon, Pancreas, Breast</td>
<td>Dalenius et al. (2007), Li et al. (2007), Lim et al. (2009)</td>
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<td>CD133</td>
<td>Glioblastoma, Medulloblastoma, Brain, Colon, Pancreas, Lung, Ovarian</td>
<td>Singh et al. (2004), Singh et al. (2004), Zhao et al. (2006), O’Brien et al. (2007), Herrmann et al. (2007), Tirnko et al. (2009), Kawasaki et al. (2009)</td>
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<tr>
<td>ALDH1</td>
<td>Endometrial, Breast, Colon, Lung, Liver</td>
<td>Rutella et al. (2009), Giresiter et al. (2007), Huang et al. (2009), Jiang et al. (2009), Schatton et al. (2008), Wu et al. (2007), Friel et al. (2008), Szotek et al. (2006)</td>
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tumour (Fillmore & Kuperwasser 2007, Visvader & Lindeman 2008). The exact function of these markers is yet to be determined, and they may be involved in essential stem cell functions such as self-renewal, although this is unlikely given that they have also present on more differentiated cells (Visvader & Lindeman 2008). Other markers to intracellular targets have been useful in confirming CSC activity of a subpopulation of cancer cells. An example is Musashi-1, which appears to mark ASC in the intestine and neural system and may be useful as a confirmatory marker in subpopulations of cells obtained using specific cell surface markers (Pilkington 2005).

**CD133 as a cancer stem cell marker**

A commonly investigated potential CSC marker is CD133 or prominin-1, a 120 kDa five transmembrane domain cell surface glycoprotein that may be involved in cell–cell interactions and mature organ homeostasis (Ricci-Vitiani et al. 2007, Klonisch et al. 2008, Ferrandina et al. 2009). CD133 has been identified on 0–42% of human colon, ovarian and prostate cancer cells, specifically localising to the luminal surface of colon cancer epithelial cells (Collins et al. 2005, Dalera et al. 2007, O'Brien et al. 2007, Ricci-Vitiani et al. 2007, Ferrandina et al. 2008, Horst et al. 2008, Choi et al. 2009, Chu et al. 2009). Non-adherent colon cancer cells cultured as spheres express CD133 and generate tumours when transplanted into NOD/severe combined immunodeficiency (SCID) mice even after 1 year of sphere culture (Ricci-Vitiani et al. 2007, Vermeulen et al. 2008). It is not clear whether these spheres arose from CSCs or progenitor cells; however, when the spheres differentiated, they stopped expressing CD133 and lost their ability to form tumours in vivo (Ricci-Vitiani et al. 2007, Vermeulen et al. 2008). Furthermore, CD133+ ovarian cancer cells were more efficient at forming clones and proliferated more extensively than the CD133− population (Ferrandina et al. 2008), and the ovarian cancer cell lines, A2780 and PEO1, and the CD133+ cells were more resistant to chemotherapeutic drugs, upregulated anti-apoptotic and development genes, and downregulated death cascade genes, in comparison to the CD133− population (Baba et al. 2008). When transplanted into mice, as few as 100 CD133+ cells were isolated from primary and metastatic colon and brain cancers, and ovarian cancer cell lines initiated serially transplantable tumours with similar historioarchitecture and differentiation into CD133+ and CD133− cells in a similar ratio as the parent tumour (Singh et al. 2004, O'Brien et al. 2007, Ricci-Vitiani et al. 2007, Baba et al. 2008). In contrast, many more CD133+ cells were required to initiate tumours that formed at a much slower rate and were smaller than those initiated by CD133− cells (Singh et al. 2004, O'Brien et al. 2007, Ricci-Vitiani et al. 2007, Baba et al. 2008). Importantly, not all CD133+ cells were able to initiate tumorigenesis (O'Brien et al. 2007), and not all cancers expressed CD133, indicating that it may not mark the CSCs in all types of cancer (Dalera et al. 2007, Choi et al. 2009, Chu et al. 2009, Ferrandina et al. 2009). In some cases of colon cancer, CD133 was correlated with more advanced stage tumours, suggesting that it may be a marker for metastasis (Choi et al. 2009); however, in ovarian cancer, the expression of CD133 decreased with metastasis or was unrelated to tumour stage (Ferrandina et al. 2008, 2009).

**CD44 as a cancer stem cell marker**

The hyaluronan receptor, CD44, is a surface glycoprotein with many signalling functions (Patrawala et al. 2006, Klonisch et al. 2008) that appears to be expressed on populations enriched for CSCs in human breast, prostate, ovarian and colon cancers. Similar to CD133, spheres generated from unsorted ovarian and colon cancer cells, and those from prostate cancer cell lines expressed CD44 (Patrawala et al. 2006, Vermeulen et al. 2008, Zhang et al. 2008). These spheres were capable of undergoing self-renewing divisions in vitro and expressed stem cell pluripotency markers including OCT-4, NANOG and BMI-1 (Patrawala et al. 2006, Vermeulen et al. 2008; Zhang et al. 2008). With differentiation, the spheres lost their expression of CD44 (Vermeulen et al. 2008), indicating that these cells might represent CSCs. In vitro evidence for CD44 as a marker for CSCs has been found in colon cancer and prostate cancer cell lines, where the CD44+ population is more efficient at forming large clones (Patrawala et al. 2006, Chu et al. 2009). In vivo, the CD44+ population in breast, prostate and colon cancers is more efficient at producing larger, more aggressive, serially transplantable tumours in NOD/SCID mice that recapitulated the original tumour phenotype in a shorter time frame, compared to the CD44− population (Al-Hajj et al. 2003, Dalera et al. 2007, Chu et al. 2009). However, studies have found a positive correlation with CD44 expression and colon tumour size, and increased CD44 expression with increased malignancy in a number of cancer cell lines, which may explain this association in the transplanted mice (Patrawala et al. 2006, Choi et al. 2009).

Some CSCs have been identified in different and sometimes non-overlapping subpopulations. Human breast CSCs have been identified in the CD44+CD24−/low population and in the ALDH+ fraction (Al-Hajj et al. 2003, Ginestier et al. 2007). Both sets of cells produced tumours in vivo that could be serially transplanted and recapitulated the original tumour phenotype (Al-Hajj et al. 2003, Ginestier et al. 2007). Interestingly, only 0.1–1.2% of the cells have overlapping marker expression and these cells are highly tumorigenic (Ginestier et al. 2007). The reason for this distinction may be because they examined different tumour subtypes and
the Al-Hajj investigation focused on metastatic rather than primary breast cancers. Alternatively, the combined ALDH+ CD44+ CD24low population may represent the true CSC, with the ALDH+ CD44+ CD24−/low and ALDH+ non-CD44+ CD24−/low populations representing more differentiated progenitors that retain some capacity to proliferate in vivo (Ginestier et al. 2007). Similarly, the CD133+ and Lgr5+ fractions appear to locate to different subpopulations in mouse colon cancer (Becker et al. 2008). Differences in CSC subpopulations both within the same tissue and between tissues may be related to culture effects, the types of cancers examined (metastatic versus primary), the ethnicity of the patients, sorting technologies (MACS versus FACS) and location of the xenograft.

Cancer stem cells and metastasis

Cancer cells that have not developed into new growths have been noted at sites distant from the original tumour. These cancer cells are often identified by pathologists based on their abnormal karyotype, or other cancer cell morphological feature. These cells may not have had time to establish a tumour due to the efficiency in their removal by the immune system, lack of blood supply or their intrinsic lack of tumour initiation capability (Southam & Brunschwig 1961, Fidler & Kripke 1977, Clark et al. 2000, Rey & Criere 2001). There are two theories regarding metastasis and the CSC. The first is that the CSC is the only cell with the traits required to establish a tumour at another site, and thus needs to escape the original tumour to establish metastasis elsewhere. Evidence for the role of CSC in establishing metastasis comes from breast cancer studies, where metastatic cells expressing the CSC phenotype, CD44+ CD24−/low, formed self-renewing mammospheres, gland-like structures in soft agar, and gave rise to differentiated progeny including CD44highCD24low cells at a higher efficiency than non-metastatic cells (Al-Hajj et al. 2003, Thani et al. 2008). The second theory postulates that CSCs and metastatic cells are different, that the metastatic cell travels to a distant site to establish a niche and then signals to the CSC to travel and take residence at the new site and initiate a metastatic tumour. This is evidenced by the increased number of CD24+ cells in distant metastases of human breast cancer (Visvader & Lindeman 2008). It has also been suggested that the CD44+ CD24−/low cells metastasize and then alter their phenotype in response to the new environment.

Cancer stem cells as prognostic indicators

While the CSC model is still controversial (Kelly et al. 2007), it is important to note that other cells may also play key roles in the development and progression of cancer (Visvader & Lindeman 2008, Rosen & Jordan 2009). If CSC surface markers relate to functional characteristics, it seems likely that the same cancer subtypes with similar molecular characteristics between patients will be initiated by similar populations of CSCs. A correlation between putative stem cell markers and poor prognosis has been observed in some cancers (Patrawala et al. 2006, Ginestier et al. 2007, Horst et al. 2008, Visvader & Lindeman 2008, Zeppernick et al. 2008); however, no statistically significant correlation between progression-free survival and CSC number has been observed in breast cancer (Abraham et al. 2005, Kern & Shibata 2007).

Cancer stem cells as targets for novel therapeutics

In cancers with strong evidence for CSCs, a new cellular target is available for drug development that focuses on eradicating the CSCs to prevent recurrence. Combining these drugs with traditional cytotoxic agents targeting the remaining cancer cells (Kern & Shibata 2007) will prevent them mutating, acquiring self-renewal properties and becoming CSCs themselves. Similarly, it will be necessary to ensure terminal differentiation of all CSCs when using differentiation induction therapies, such as ATRA for acute promyelocytic leukaemia (Petrie et al. 2009). Targeting the CSCs may be especially important in higher grade and advanced tumours, where evidence indicates that the frequency of CSCs is greater in that lower grade and lower staged tumours (Visvader & Lindeman 2008, Rosen & Jordan 2009), and also in tumours where the majority of tumour cells have already been eradicated (Kern & Shibata 2007). It will be important that new treatment options spare normal stem cells or are delivered in a targeted manner that avoids normal stem cell exposure.

Endometrial carcinoma

Incidence

Endometrial cancer (EC) is the most common gynaecological malignancy affecting women in the western world (National Institute of Health 2009). In 2009, it is expected that there will be 42,160 new cases diagnosed and 7780 deaths from the disease in USA (National Institute of Health 2009). It has an incidence rate of 23.3 per 100,000 women, and the average age at diagnosis is 62 years (Bethesda 2008). The 5-year survival rate for EC is 82.9%, mainly because cases are diagnosed at an early stage due to the readily detectable symptom of abnormal uterine bleeding in post- and peri-menopausal women (Bethesda 2008).

Histological classification of endometrial carcinomas

There are a number of histological types of EC: endometrioid carcinoma, mucinous adenocarcinoma, papillary serous adenocarcinoma, clear cell
adenocarcinoma, undifferentiated carcinoma and mixed carcinoma (Creasman et al. 2006). Around 10% of ECs are non-sp=rdastic mostly associated with hereditary non-polyposis colorectal cancer (Ryan et al. 2005, Bansal et al. 2009). In this condition, there are abnormalities in DNA mismatch repair genes that eventually results in microsatellite instability (MSI; Ryan et al. 2005). Women with this genetic susceptibility have a tenfold higher lifetime risk of developing EC in comparison to the general population (Ryan et al. 2005). The remaining 90% of EC cases are considered sporadic (Ryan et al. 2005, Bansal et al. 2009), with various genetic mutations being identified.

The International Federation of Gynecology and Obstetrics (FIGO) has classified EC into three grades on the basis of morphology (FIGO & Obstetrics 1989). Grade 1 tumours are neoplasms with ≤ 5% non-squamous components, a non-mucinous solid growth pattern (FIGO & Obstetrics 1989, Creasman et al. 2006, Zaino 2009). In grade 2 tumours, 5–50% of the neoplasia is non-squamous and non-mucinous, and in grade 3 tumours, >50% of the tumour is a solid mass (FIGO & Obstetrics 1989, Zaino 2009). In general, grade 1 tumours are well-differentiated, grade 2 moderately differentiated and grade 3 poorly differentiated (Creasman et al. 2006). FIGO grading increases by 1 when nuclear atypia appears inappropriate for the architecture of the grade (e.g. grade 1 becomes grade 2), and when adenocarcinomas contain squamous differentiation, the cells are graded according to the nuclear grade of the glandular component (Creasman et al. 2006). Serous, clear cell and pure squamous cancers are designated as grade 3 by convention due to their poor prognosis (Zaino 2009).

Types of endometrial carcinoma
In 1983, Bokhman performed a large study over 20 years and classified sporadic ECs into two types based on their aetiology and clinical behaviour (Bansal et al. 2009). This classification has been confirmed on the basis of genetic mutation differences between the two types. Type I ECs have an endometrioid histology and account for 70–85% of sporadic cases (Creasman et al. 2006, Di Cristofano & Ellison 2007, Bansal et al. 2009). They have a favourable prognosis as they are often diagnosed at an early stage and are of a low grade (Bokhman 1983, Creasman et al. 2006, Di Cristofano & Ellison 2007, Bansal et al. 2009). These tumours often arise from endometrial hyperplasia in a setting of unopposed oestrogen in peri- and post-menopausal women and commonly express oestrogen and progesterone receptors (Bokhman 1983, Di Cristofano & Ellison 2007, Bansal et al. 2009). Mutations in PTEN, β-catenin, K-RAS and MSI are molecular lesions associated with type I EC (Di Cristofano & Ellison 2007).

Type II tumours account for 10–20% of sporadic ECs and often have a serous papillary or clear cell histology (Bokhman 1983, Prat et al. 2007, Bansal et al. 2009). They tend to be composed of markedly atypical cells that grow in papillary, glandular or solid patterns (Di Cristofano & Ellison 2007). Type II tumours arise in a background of atrophic post-menopausal endometrium independent of oestrogen and may be preceded by endometrial intraepithelial carcinoma (EIC; Bokhman 1983, Di Cristofano & Ellison 2007, Prat et al. 2007, Bansal et al. 2009). EIC is composed of cells that are indistinguishable from carcinoma cells, but confined to endometrial epithelium, without invasion into the underlying stroma (Di Cristofano & Ellison 2007, Prat et al. 2007). Type II tumours have a poor prognosis as they tend to spread from the site of origin early in the development of the disease (Bokhman 1983, Prat et al. 2007, Bansal et al. 2009). Mutations in TP53 and HER-2/neu genes and aneuploidy are commonly found in type II cancers, whereas mutations in K-RAS, PTEN or MSI are relatively uncommon, suggesting different molecular pathways for tumourigenesis in the two types of EC (Di Cristofano & Ellison 2007).

Cancer stem cells in endometrial carcinoma
Given that a rare population of epithelial stem/progenitor cells has been identified in human endometrium (Chan et al. 2004, Gargett et al. 2009), it is possible that these cells or their progeny may be the source of the putative CSCs that may initiate and maintain EC (Fig. 1; Gargett et al. 2008). The first evidence for a stem cell origin of EC was suggested in 1997 in a study on a uterine carcinomas derived cell line (Gorai et al. 1997). In this study, colony-initiating cells grew for over 50 serial passages and were composed of cells with columnar, small epithelial, moderately sized or large epithelial-like, malignant tumour giant and spindle-shaped morphologies, similar to those found in the original cell line (Gorai et al. 1997). These highly proliferative clonal cells expressed immunohistochemical and molecular markers consistent with their parental tissue, recapitulated the tumour phenotype in vitro and were considered stem cells responsible for propagating the cell line (Gorai et al. 1997). While these results were promising, they were not performed on freshly isolated EC cells, or even a pure EC cell line.

Identification of CSC activity in human EC samples
Recently, freshly isolated cells from EC tissues have been investigated for CSC properties (Hubbard et al. 2009). A small population of freshly isolated EC cells has the capacity to initiate clones that undergo self-renewing cell divisions and initiate tumours in vivo that can be
serially passaged, demonstrating self-renewal, proliferation and differentiation abilities of the potential CSCs.

Less than 1% of single EC cells from all grades and both types of EC initiated clones in vitro (Hubbard et al. 2009). As a range of clone sizes were observed, more differentiated progenitor cells may have also contributed to the observed cloning efficiency (Hubbard et al. 2009). This demonstrated that only rare cells within EC are able to proliferate and initiate large colonies, conforming to the CSC hypothesis.

Heterogeneous proliferative potential of EC cells was further confirmed in tumorigenicity studies. EC cells were subcutaneously transplanted in NOD/SCID mice in limiting dilution, but not all dilutions initiated tumours, a phenomenon observed in all grades and both types of EC (Fig. 2; Hubbard et al. 2009). The tumours re-capitulated the original tumour histology and differentiation marker expression, including ERα, PR, vimentin and cytokeratin (Hubbard et al. 2009). Type I EC cells also formed tumours when injected subcutaneously; however, larger numbers of cells were required to initiate tumour formation (Friel et al. 2008, Hubbard et al. 2009). This is probably related to the superior blood supply from kidney tissue into the xenograft as has been observed in other tumours (Visvader & Lindeman 2008).

The ability of some EC cells to initiate tumours supports a CSC origin of EC, and these cells may be similar to the original cells that gave rise to the tumour in the patient. Colony-forming units/cells from all grades and both types of EC underwent several rounds of serial subcloning in vitro, indicating substantial self-renewal, an essential CSC property (Hubbard et al. 2009). A trend of increasing self-renewal ability was observed with increasing tumour aggressiveness in this study (Hubbard et al. 2009). Secondary clones also expressed several self-renewal genes, including BMI-1, β-CATENIN, SOX-2 and NANOG (Hubbard et al. 2009). Also, primary subcutaneous tumours established from freshly isolated xenografted EC cells were serially passaged up to five times in immunocompromised mice, maintaining the original tumour morphology and phenotype when examined by immunohistochemistry, demonstrating the CSC traits of self-renewal and differentiation in vivo (Friel et al. 2008, Hubbard et al. 2009). While type II tumours appear to initiate tumours more efficiently (Hubbard et al. 2009), no trend of increasing self-renewal with increasing tumour aggressiveness was observed in vivo, perhaps due to low sample sizes of the investigations.

Together, these data from in vitro and in vivo studies on EC and EC cell lines provide substantial evidence for a CSC origin for EC. However, these studies are generally retrospective and a cell surface marker or combination of markers for the isolation of EC CSCs is required, so that the CSCs can be isolated, characterised and their role in the development of the disease was studied.

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**Figure 1** Hypothesised role of cancer stem cells in the development of endometrial carcinoma. (A) Normal endometrial gland with a potential epithelial stem progenitor cell located at the base of the gland. (B) Epithelial progenitor cell acquires genetic mutations resulting in the development of the CSCs in either type I (left, orange) or type II (right, dark blue) EC. Expansion of the CSC clone producing heterogeneous neoplastic epithelial cells in type I (C) and type II (D) ECs, comprising a small number of CSCs (C, orange; D, dark blue) and numerous differentiated tumour cells (C, light orange; D, light blue).
Side population cells as EC stem cells

Side population (SP) cells are cells capable of expelling the Hoechst 33342 dye, often due to the presence of plasma membrane ABCG2 transporters. A recent study investigating CSCs in EC has examined several EC cell lines and four high grade ECs for the presence of SP cells (Friel et al. 2008). In the AN3CA and Ishikawa, but not the SKUT-2, HEC-1-A and HEC-1-B EC cell lines, and in four EC cell suspensions, rare (< 3.4% of cells sorted by FACS) SP cells were detected (Friel et al. 2008; Fig. 3). SP cells from the cell lines demonstrated several CSC traits, including slow growth rate in vitro, demonstrated by a higher percentage of cells in the G1 phase of the cell cycle than the main population (MP), suggesting that SP cells cycled less frequently than the majority of the tumour cells (Friel et al. 2008). Another trait was the resistance of SP cells to paclitaxel, suggestive of the presence of drug transporters, such as ABCG2. In vitro culture of the SP cells from the cell lines recapitulated the original SP:MP cellular ratio, suggesting self-renewal and differentiation potential of cells in the SP fraction (Friel et al. 2008). Finally, SP cells, but not MP cells, from the cell lines and the four EC tissue initiated tumours in NOD/SCID mice when injected subcutaneously (Friel et al. 2008). However, it is not known whether the resultant tumours expressed typical EC markers (e.g., ERα, PR, cytokeratin, vimentin), nor was it determined whether the SP-initiated tumours contained both SP and MP cells, indicating that differentiation and self-renewal of the SP cells had occurred in vivo. However, this study does provide evidence for a CSC origin of EC.

Markers identifying EC stem cells

Molecular and cell surface markers of CSCs have recently been identified in EC tissues. Musashi-1, a RNA-binding protein expressed by epithelial progenitors of the gastric mucosa, intestine, mammary glands, epidermis and hair follicles, is associated with self-renewal functioning of ASCs (Götte et al. 2008, Sureban et al. 2008). In xenografted colon adenocarcinomas, siRNA-mediated knockdown of Musashi-1 reduced cellular proliferation and increased apoptosis (Sureban et al. 2008), indicating that the potential CSCs maintaining the cancer were unable to function in the absence of Musashi-1. Musashi-1-expressing single cells were found in sections of EC samples. It was hypothesised that these were EC progenitor or CSCs.
However, functional assays of CSC activity (Gargett 2007) are required to confirm that Musashi-1 identifies CSC in human EC.

**CD133 as a marker of EC stem cells**

CD133+ (referred to as CD133) is the first marker used to identify and prospectively isolate EC stem cells (ESCs; Rutella et al. 2009). Both the CD133+ and CD133- sorted fractions of EC single cell suspensions grew in culture, giving rise to CD133+ and CD133- cells, although the CD133+ fraction had a higher proliferation rate (Rutella et al. 2009). The CD133+ fraction produced single cell-derived colonies at greater efficiency than the CD133- fraction (Rutella et al. 2009), and the CD133+ cells were more resistant to the cytotoxic effects of cisplatin and paclitaxel than the CD133- fraction (Rutella et al. 2009). Interestingly, both populations were equally sensitive to doxorubicin, indicating that this drug may be effective in treating EC. Furthermore, as EC is an oestrogen-dependent disease, the proliferation potential between the populations was compared in the presence of oestradiol (Rutella et al. 2009), with the CD133+ population showing increased proliferation, whereas the CD133- population did not respond. The CD133+ population also had a higher expression of ERα than the CD133- population (Rutella et al. 2009). However, it is not known whether the CD133+ population includes niche cells which may also express ER and relay proliferative signals to the putative ESCs. In normal mouse endometrium, the potential epithelial progenitor cell (label retaining cell) did not express ERα, but the neighbouring niche cells did (Chan & Gargett 2006), indicating that normal progenitor cells respond indirectly to oestradiol, via signals provided by niche cells (Chan & Gargett 2006). However, it is not known whether ESCs will have similar properties to mouse endometrial epithelial label retaining cells. The case may be different in human cancer as it is not known from which cells the CSC arises. Also, the growth kinetics and changes in CD133 expression of the unsorted populations were not compared, and thus it is not known whether there is any interaction between the two populations. Together, these data indicate that ESCs may be located within the CD133+ population; however, evidence is still lacking.

Unsorted single EC cells subcutaneously transplanted into NOD/SCID mice formed tumours which contained an increased proportion of cells expressing CD133 (20 to 80–90%; Rutella et al. 2009), suggesting that CD133+ cells may have a greater survival potential within the mouse host. However, freshly isolated CD133+ or CD133- cells were each incapable of forming tumours *in vivo* (Rutella et al. 2009), indicating that the two fractions may interact during tumourigenesis. It is important to note that in the samples where CD133+ or CD133- cells were transplanted, tumours also failed to develop when the unsorted cells from these same samples were transplanted, potentially indicating that these tumours were devoid of TICs. It will be important to undertake additional investigations to confirm this possibility. Furthermore, both CD133+ and CD133- fractions isolated from primary xenografts derived from unsorted cells formed tumours of comparable sizes when re-transplanted into NOD/SCID mice. This apparent contradiction to the differential *in vitro* abilities of the CD133+ and CD133- cells indicates that TICs may be present in both the CD133+ and CD133- fractions, especially after the cells have had the opportunity to adapt to the mouse kidney microenvironment (Rutella et al. 2009). It is also possible that CD133 may be expressed on non-tumour-initiating progenitor or transit-amplifying cells unable to proliferate *in vitro*. This study highlights the importance of undertaking a range of CSC assays to confirm the utility of potential markers to enrich for CSC.

Interestingly, CD133 expression was not correlated with tumour grade or CA-125 serum levels, but with endometroid subtype, as the tumours were mainly early stage, and accordingly CD133 expression was not found in patients with lymph node metastases (Rutella et al. 2009). Further work is required to confirm whether CD133 is a marker present on ECs and also needs to address the apparent differences between the *in vitro* and *in vivo* experiments.

With the continued investigation of CD133 as a marker of ECSC and the future discovery of other markers for ECSCs, it is hoped that ECSCs can be isolated and characterised, and that their role in the development of human EC was further investigated. This knowledge opens the way for the development of new treatment modalities that target the CSCs, but spare the remaining normal endometrial stem/progenitor cells and other cells. Such treatments will be particularly useful for early-stage and pre-menopausal EC candidates where the uterus may be conserved, and for late-stage cases where hysterectomy is not curative and current treatments target the bulk tumour cells rather than CSCs.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Adult Stem Cells in the Human Endometrium
Caroline E. Gargett, Irene Carvelló, Sonya Hubbard, and Carlos Simón

INTRODUCTION
The human endometrium is a dynamic remodeling tissue undergoing more than 400 cycles of regeneration, differentiation, and shedding during a woman’s reproductive years. The coordinated and sequential actions of estrogen and progesterone direct these major remodeling events to prepare the endometrium for blastocyst implantation. Endometrial regeneration also follows parturition, almost complete curettage and occurs in postmenopausal women taking estrogen replacement therapy. Adult stem/progenitor cells are likely responsible for endometrial regeneration. This chapter will review the evidence available to date for the existence of adult stem/progenitor cells in human endometrium. It will detail the functional approaches that have been used to identify candidate endometrial epithelial and mesenchymal stem/progenitor cells, in particular, cell cloning and side population (SP) studies. The importance of in vivo studies that demonstrate the ability to reconstruct endometrial tissue from isolated cell populations will also be emphasized. Identification of markers that distinguish endometrial epithelial and mesenchymal stem/progenitor cells from their more differentiated progeny is essential to progress this newly developing field of research. Approaches being undertaken to identify markers for endometrial mesenchymal stem/progenitor cells will be outlined, including the recent discovery that co-expression of two markers, CD146 and platelet-derived growth factor (PDGF) receptor-β, partially purifies human endometrial mesenchymal stem/progenitor cells. This CD146⁺ PDGF-Rβ⁺ population has a surface phenotype similar to bone marrow and fat mesenchymal stem cells (MSC), and demonstrates multipotency by their capacity to undergo multilineage differentiation into fat, cartilage, bone, and smooth muscle. They have a perivascular location in the functionalis and basalis layers of the endometrium. Application of these fundamental studies to the current knowledge on the pathophysiology of a variety of common gynecological diseases associated with abnormal endometrial proliferation, including endometrial cancer, endometriosis, and adenomyosis will also be discussed. Mention will be made of the possible use of endometrial stem/progenitor cells in autologous tissue engineering applications relevant to urogynecology. Finally, the future directions of human endometrial stem/progenitor cell research will be suggested.

REGENERATIVE CAPACITY OF HUMAN ENDOMETRIUM
In humans, the failure of an embryo to implant into the receptive endometrium at the appropriate time results in the sloughing off of the functional layer of the endometrial lining, which is regenerated in the next menstrual cycle (Fig. 1). Thus, the human endometrium is a dynamic remodeling tissue undergoing more than 400 cycles of regeneration, differentiation, and shedding during a woman’s reproductive years (2-4). The first stage of endometrial repair involves migration of epithelial cells from protruding stumps of basal glands over the demided surface within 48 hours of shedding (2,3). Endometrial repair does not appear to require estrogen as this process occurs while circulating estrogen levels are very low and when epithelial cells lack estrogen receptor-α (ERα) expression (3). As estrogen levels rise during the proliferative stage, ERα and progesterone receptors (PR) are induced in the epithelium and stroma. The functionalis mucosa grows 4 to 10 mm within 4 to 10 days (4), with extensive proliferation of glandular epithelial cells and, to a lesser extent, stromal cells (Fig. 1) (5-7). Following ovulation, proliferation gradually ceases and the estrogen-primed functionalis commence differentiation under the influence of progesterone, which suppresses functionalis, but not basalis ERα and PR (8,9). The differentiating endometrial glands secrete large quantities of glycogen and histotrophic secretory products in preparation for an implanting blastocyst (8,10,11). PR persist on stromal cells in the functionalis, which proliferate and
differentiate into predecidual cells surrounding around spiral arterioles and beneath the luminal epithelium. Endometrial differentiation is also accompanied by dramatic changes in gene expression profiles (12). When implantation fails to occur, stromal decidualization becomes a terminal differentiation. The demise of the ovarian corpus luteum and subsequent fall in circulating estrogen and progesterone levels trigger menstruation, and the functionalis is shed (Fig. 1) (9,13). The remaining basalis layer of the endometrium, which is relatively insensitive to sex steroid hormone actions and undergoes little proliferation or differentiation (8), acts as a germinal compartment providing a source of endometrial cells for regenerating the new functionalis in the subsequent cycle (5,14).

When menstrual cycles cease at menopause, the endometrium becomes very thin and atrophic, containing a few glands in a stroma resembling the basalis (15,16). Similarly, women taking oral contraceptive pills (OCP) do not exhibit cyclic changes in circulating sex steroid hormones and their endometrium does not undergo cyclical growth, differentiation, and regression. Histologically, OCP endometrium appears inactive, with similar morphology to postmenopausal endometrium. However, when postmenopausal women take estrogen replacement therapy or women cease OCP medication, their endometrium regenerates. Endometrial regeneration also follows parturition, extensive resection, and unsuccessful endometrial ablation therapies for menorrhagia (17).

ENDOMETRIAL STEM/PROGENITOR CELL HYPOTHESIS
The monthly regeneration of the human endometrium and its reconstruction following parturition are at least equivalent to the level of cellular turnover that occurs in other highly regenerative organs such as blood-forming tissue of the bone marrow, epidermis, and intestinal epithelium (17). In these regenerative tissues, adult or somatic stem cells, responsible for provision of replacement cells to maintain tissue homeostasis, have been identified. The concept that basalis endometrium harbors stem/progenitor cells responsible for the remarkable regenerative capacity of endometrium was proposed many years ago (14,18,19). Indirect evidence for the existence of adult stem/progenitor cells in the endometrium has accumulated over the intervening years (reviewed in Ref. 17). Attempts to isolate, characterize, and locate endometrial stem/progenitor cells have recently been undertaken as experimental approaches to identify adult stem cells in other tissues (17), resulting in the identification of rare populations of epithelial stem/progenitor cells and mesenchymal stem-like cells in human endometrium.
Adult Stem Cells and Their Properties
Somatic stem cells are undifferentiated cells present in most adult tissues. Their rarity, the lack of distinguishing morphological features, and current lack of known specific markers make them difficult to identify in many tissues. Adult stem cells are defined by their functions: high proliferative potential, self-renewal, and differentiation into one or more lineages (20). The retention of a DNA synthesis label [bromodeoxyuridine (BrdU)] for prolonged periods of time is another property of adult stem cells, since, paradoxically, they proliferate less frequently than their daughter cells (21). Adult stem cells maintain tissue homeostasis by providing replacement cells in regenerating tissues, in routine cellular turnover, and for repair after acute injury (22,23). The balance between adult stem cell self-renewal and differentiation is strictly regulated by the stem cell niche, comprising the adult stem cells, surrounding niche cell(s) and extracellular matrix, to ensure an appropriate balance between stem cell replacement and provision of sufficient differentiated mature cells to maintain tissue homeostasis for organ function (22).

Endometrial Epithelial Stem/Progenitor Cells
Cell Cloning Studies
The first published evidence for the existence of endometrial epithelial stem/progenitor cells in human endometrium came from cell cloning studies, where single-cell suspensions were seeded at cloning density in culture (24). Rare clonogenic epithelial cells were identified in normal cycling and inactive perimenopausal endometrium and in endometrium of women on oral contraceptives (24,25), suggesting that clonogenic epithelial cells may be responsible for regenerating glands in cycling and atrophic endometrium (26). These studies found that 0.22% of human endometrial epithelial cells had colony-forming unit (CFU) activity. Two types of CFU formed: large (0.09%) and small (0.14%), leading to the hypothesis that large CFU are initiated by stem/progenitor cells possibly located at the base of the glands in the basalis (Fig. 2). Small CFU are possibly initiated by more differentiated transit amplifying cells, likely located in the functionalis layer and responsible for the extensive proliferation observed in the first half of the menstrual cycle (17,24,27). Differential expression of epithelial markers was noted between large

Figure 2: The relationship of endometrial colony-forming cells to the hierarchical model for stem cell differentiation. Stem cells have the capacity to self-renew and replace themselves as well as differentiate into committed progenitors through asymmetric cell divisions. Progenitors proliferate and give rise to more differentiated rapidly proliferating transit amplifying cells, which finally differentiate to produce a large number of terminally differentiated functional cells with no capacity for proliferation. We postulate that the large colonies are initiated by putative stem/progenitor cells and the small colonies by putative transit amplifying cells. Source: From Ref. 24.
and small CFU. Small CFU expressed epithelial differentiation markers, cytokeratin, epithelial cell adhesion molecule (EpCAM), and α6-integrin, but only the latter was expressed in cells of large CFU, which comprised small cells with a high nuclear/cytoplasmic ratio, suggesting an undifferentiated phenotype (24). The percentage of endometrial epithelial CFU did not vary with menstrual cycle stage, indicating their continued presence in human endometrium (25). The growth factor requirements of human endometrial epithelial CFU have been characterized in serum-free culture conditions. Fibroblast feeder layers were necessary for serum-free growth, indicating the importance of surrounding stromal cells, which are likely the niche cells that regulate clonogenic epithelial cell fate decisions. Epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and PDGF-BB were requisite growth factors for human endometrial epithelial CFU activity, while insulin-like growth factor-1 (IGF-1), leukemia growth factor (LIF), stem cell factor, and hepatocyte growth factor were weakly supportive and basic fibroblast growth factor (bFGF) was without effect (24). This data suggests that EGF receptors are present on endometrial epithelial CFU. However, it is likely that PDGF-BB exerted its mitogenic effects on epithelial CFU indirectly via the PDGF receptor-β on the fibroblast feeder cells (27).

Side Population Cells
Another approach to the identification and isolation of adult stem cells in adult tissues or organs in the absence of known specific markers for these cells is to use the Hoechst dye exclusion method. A specific SP displaying low red and low blue fluorescence can be identified by dual-wavelength flow cytometry after incubating the target cells with the DNA-binding dye Hoechst 33342 (28). This phenomenon is due to the expression of a cell membrane transporter related to immature cells, breast cancer resistance protein 1 (BCRP) (29). The SP phenotype is thought to be a universal marker of adult stem cells and has been used to isolate adult stem cells from many adult tissues (30).

Recently, an epithelial SP population was identified in human endometrium (31,32). The mean percentage of SP cells in the epithelial fraction was 0.21% during the menstrual phase, 0.15% during the proliferative phase, and 0.07% in the secretory phase (31). After five months in 3D culture conditions, the endometrial epithelial SP cells formed gland-like CD9-expressing structures, indicating their ability to differentiate (31). A study by Cervero et al. corroborates the existence of epithelial SP cells in the human endometrial epithelium (Fig. 3) (32). Furthermore, the isolated SP cells expressed typical undifferentiation markers such as c-KIT and OCT-4 at the mRNA level, and they had telomerase activity intermediate between human embryonic stem cells and differentiated cells. Phenotypic analysis of these SP cells revealed that more than 50% were CD34+ (mesenchymal and endometrial stromal cell marker) and negative for CD34 (endothelial) and hemopoietic stem cell marker) and CD45 (leukocyte marker) expression (32), suggesting that they are of mesenchymal origin. Clone-negative and in vivo endometrial tissue reconstitution studies are necessary to further demonstrate the differentiation potential of the SP cells isolated from the epithelial fraction of human endometrium (1).

Figure 3 Identification of SP cells in the human endometrium. (A) Typical diagram showing the distribution of the Hoechst-retaining cells in a FACS histogram. (B) SP cells obtained from human endometrium. (C) Human endometrial cells treated with verapamil where SP region disappears.
Label-Retaining Cells
Label-retaining cells (LRC) have been identified as candidate adult stem cells in vivo in mouse endometrium (33-35). The LRC approach identifies adult stem cells by their quiescent, slowly cycling nature, since they only undergo cell division during tissue turnover to initiate replacement of lost cells. It is based on pulse labeling the majority of tissue cells with a DNA synthesis label (BrdU) during a time when adult stem cells are proliferating and a subsequent chase of the label over long periods of time. Slow-cycling stem cells retain the label, while rapidly dividing transit amplifying cells dilute the label to undetectable levels. Immunohistochemistry localizes BrdU+ LRC, revealing their location and the stem cell niche. Only one study to date has demonstrated epithelial LRC in the mouse endometrium (33). During a chase period of 56 days, the BrdU label diluted rapidly because of extensive proliferation of luminal epithelium as nascent glands developed during neonatal and prepubertal endometrial growth and during subsequent estrus cycles. Epithelial LRC, comprising 3% of mouse endometrial epithelial cells, were observed as separate cells in the luminal but not glandular epithelium, suggesting that luminal epithelial stem/progenitor cells are responsible for the growth of glands during development and in the cycling adult mouse (33). They may also have an important role in regenerating luminal epithelium, which undergoes substantial proliferation and apoptosis during the estrus cycle (36).

Endometrial Stromal/Mesenchymal Stem-Like Cells
Cell Cloning Studies
Several published studies support the existence of stromal/mesenchymal stem-like cells in human endometrium. Initial studies identified a small population (1.25%) of freshly isolated human endometrial stromal cells possessing colony-forming ability (24). These CFU are not only retained in culture but their proportion increases to 15% of stromal cells after prior expansion in culture at normal seeding densities (37). Similar to epithelial CFU, two types of stromal CFU formed from freshly isolated cells, with only 0.02% of stromal cells initiating large CFU, supporting a stromal cellular hierarchy hypothesized to exist in human endometrium (17). Both large and small stromal colonies expressed fibroblast markers, with some cells expressing α smooth muscle actin (αSMA), indicative of myofibroblast differentiation. Four growth factors supported stromal CFU in serum-free cultures: bFGF, EGF, TGFα, and PDGF-BB (24,25), indicating that stromal CFU are different from epithelial CFU and that there are separate epithelial and stromal adult stem cells in human endometrium.

Multilineage Differentiation
A key adult stem cell property is the ability to undergo multilineage differentiation. MSC have been identified in bone marrow and fat, and these cells differentiate into at least three mesenchymal lineages in vitro under appropriate induction conditions (38). Various human endometrial stromal cell populations can be induced to differentiate into one or more of these mesenchymal lineages, suggesting that MSC-like cells may reside in human endometrium. Various studies have demonstrated that some stromal cells in a heterogeneous population of cultured human endometrial stromal cells differentiate into fat or chondrocyte lineages (37,39). Since mixed populations of stromal cells were examined, these studies were unable to determine whether individual stromal cells are multipotent. Similarly, a recent study examining the differentiation potential of a CD146+PDGF-Rβ+ fraction of endometrial stromal cells enriched eightfold for human endometrial stromal CFU showed that these cells differentiated into four mesenchymal lineages: adipogenic, chondrogenic, osteoblastic, and myogenic (40). Although the CD146+PDGF-Rβ+ cells are a partially purified population, the multilineage capacity of individual cells was not examined. Recently, the differentiation capacity of single human endometrial stromal cells was demonstrated proving that human endometrium contains truly multipotent MSC (1).

Side Population Cells
Rare SP cells were also identified in the human endometrial stromal compartment. The greatest number (3.9%) was observed during the menstrual phase, probably because of the
mobilization of this population at this specific time (31). Much lower percentages of stromal SP cells were identified in the proliferative (0.06%) and secretory (0.1%) phases. The expression of stromal markers CD13 and vimentin was confirmed in the isolated stromal SP cells, which did not express E-cadherin or CD9, markers of epithelial cells. The human endometrial stromal SP cells proliferated slowly in long-term cultures and differentiated into stromal-like clusters after six months in 3D matrix culture. Cervello et al. confirmed the presence of SP cells in the human endometrial stromal compartment (CD13+ cells). They found 0.01% to 1.36% SP cells per more than 50 samples. These stromal SP cells expressed the typical undifferentiation markers, c-KIT and OCT-4, suggesting a degree of undifferentiation in this subset of cells. Further phenotypic analysis for CD90 confirmed that endometrial stromal SP cells are of mesenchymal and endometrial origin (42). This data supports the hypothesis for the existence of endometrial stromal stem/progenitor cells in the human endometrium, although further studies are required to determine if stromal SP cells form CFU and express defining MSC properties such as multilineage differentiation.

SP cells have also been identified in freshly isolated human myometrial cells (43). Myometrial SP cells were CD34-CD45-, indicating that they were not hematopoietic stem cells. They were relatively undifferentiated as they expressed lower levels of ERα, PR, and smooth muscle cell markers, calponin and smoothelin, compared with main population (MP) myometrial cells, and spontaneously differentiated into mature myometrial cells expressing αSMA and calponin under hypoxic conditions (43). Some also underwent multilineage differentiation into osteogenic and adipogenic lineages when cultured in appropriate differentiation induction media. Myometrial SP cells expressed some bone marrow MSC surface markers including STRO-1, CD105, CD73, and CD106, but not CD44. Human myometrial SP cells transplanted into the uterine horns of non-obese diabetic/severe combined immunodeficient/γc-null (NOD/SCID/γc-null) (NOG) mice (44) supplemented with estrogen incorporated into myometrium and co-expressed vimentin and αSMA. These cells also induced the expression of a pregnancy marker, oxytocin receptor mRNA, in pregnant but not non-pregnant uterus of NOG mice previously transplanted with SP cells, indicating the functional capacity of the myometrial SP cells (43). This study supports the existence of myometrial MSC-like cells with ability to produce mature myometrial cells in vitro and contribute to myometrial tissue in vivo. The relationship of myometrial SP cells with MSC-like properties to endometrial SP cells or the CD166-PLGF-Rβ+ mesenchymal stem/progenitor cells identified in human endometrium is not currently known. Perhaps myometrial SP cells are derived from müllerian duct mesenchyme during fetal uterine development since the inner myometrial smooth muscle layer develops from müllerian duct mesenchyme, the endometrial stroma primordium.

Label-Retaining Cells
Candidate stromal stem/progenitor cells have been identified in mouse endometrium as stromal LRC (33-35). Between 6% and 9% of the stromal cells were identified as LRC after at least 12-weeks chase of the BrdU label. A large proportion of these were located near blood vessels close to the endometrial-myometrial junction (33,34), correlating with their postulated basalis location in human endometrium. Stromal LRC were further characterized for expression of various markers. They were not leukocytes or of bone marrow origin as they did not express CD45 (33). Some expressed ERα (35), and some expressed typical markers of undifferentiation, c-Kit and Oct-4 (36), although in another study, c-Kit was not expressed by endometrial stromal LRC (35). The continued decrease in the percentage of LRC as the chase period lengthened indicates that the LRC also undergo symmetric cell divisions in adulthood or do not necessarily retain their template DNA strands, as has been demonstrated for LRC in the intestine (45).

Menstrual Blood Stem/Progenitor Cells
There is increasing evidence that endometrial stem/progenitor cells may be shed in menstrual blood (46), although the number of samples reported to date is extremely small (47,48) and further characterization of the stem cell status of isolated cells requires verification. Menstrual blood not only contains fragments of shed endometrial functionalis,
where adult stem cells are not expected to reside, but also peripheral blood, which may contain a small number of hematopoietic stem cells, MSC, or endothelial progenitor cells. Furthermore, stem cells are rare cells in any tissue, and it is difficult to understand a physiological system dispensing with these important cells so readily each month. Nevertheless, the shedding of endometrial stem/progenitor cells into menstrual blood requires more investigation.

Endometrial Stem/Progenitor Cell Reconstitution of Endometrial Tissue In Vivo

The functional proof of the regenerative capacity of the putative endometrial adult stem cell population is the reconstitution of the original tissue in vivo. Functional endometrium has been regenerated from singly dispersed unfractionated human endometrial cell suspensions xenotransplanted beneath the kidney capsule of ovariectomized and estrogen-supplemented NOG mice lacking T, B, and natural killer cells (49). Well-organized endometrial tissue comprising glandular structures expressing typical epithelial markers such as cytokeratin and CD9, stroma positive for CD10 and CD13, and myometrial layers was reproduced (49). In this animal model, both compartments underwent typical hormone-dependent changes such as production of tortuous glands and stromal decidualization, characteristic of secretory phase, on simulation of the menstrual cycle with cyclic administration of estrogen and progesterone (49). Grafts collected after hormonal withdrawal contained large blood-filled cysts similar to red spot lesions of active endometriosis. Immunohistochemical staining of the cystic lesion revealed that the glandular structures were partly destroyed and that hemorrhage had occurred in the degenerated stroma (49). This animal model provides an excellent in vivo assay system to test whether candidate human or mouse endometrial stem/progenitor cell populations, such as the SP cells or CD146⁺PDGF-Rβ⁺ stromal cells, can reconstitute endometrial tissue (50).

Markers of Endometrial Stem/Progenitor Cells

Currently, there are no markers for endometrial epithelial stem/progenitor cells and they cannot be distinguished from their mature progeny, the pseudostratified epithelium comprising the glands and luminal epithelium.

However, MSC-like cells were recently isolated from human endometrium using co-expression of two perivascular cell markers, CD146 and PDGF receptor-β (PDGF-Rβ) (40). The fluorescent activated cell sorted (FACS)-sorted CD146⁺PDGF-Rβ⁺ subpopulation of endometrial stromal cells was enriched eightfold for CFU compared with unsorted stromal cells. That PDGF-BB supports stromal CFU activity further supports the use of PDGF-Rβ as a marker for the prospective isolation of MSC-like cells from human endometrium. These CD146⁺PDGF-Rβ⁺ cells expressed typical MSC surface markers, CD29, CD44, CD73, CD90, and CD105, and were negative for hematopoietic and endothelial markers (CD31, CD34, and CD45) (40). STRO-1, the classic marker used to prospectively isolate bone marrow MSC, was not expressed by these cells nor by clonogenic stromal CFU (42). This study also demonstrated multilineage differentiation of CD146⁺PDGF-Rβ⁺ cells into adipogenic, myogenic, chondrogenic, and osteoblastic lineages when cultured in appropriate induction media (Fig. 4). This data suggests that the CD146⁺PDGF-Rβ⁺ subpopulation of endometrial stromal cells contains MSC-like cells similar to MSC of bone marrow, fat, and dental pulp (51-53). Furthermore, confocal microscopy demonstrated that CD146 and PDGF-Rβ co-expressing cells were located perivascularly in the functionalis and basalis (Fig. 5) (40). Whether MSC-like cells in the basalis and/or functionalis are involved in regenerating endometrium awaits the identification of more specific markers. This finding also suggests that it is possible that endometrial MSC-like cells are shed during menstruation. The demonstration of endometrial tissue reconstitution and differentiation in vivo would further support the existence of MSC in human endometrium.

A number of studies have examined stem cell marker expression in human and mouse endometrium by immunotechniques. These studies, while valuable, require further analyses to validate whether the cells expressing these markers function as endometrial stem/progenitor cells (50). Unfortunately, there are no specific markers of adult stem cells, although some pluripotency genes indicate the likely presence of adult stem cells. OCT-4, a transcription
Figure 4 Multilineage differentiation of CD146-PDGFRβ human endometrial stromal cells and their perivascular location. FACS-sorted CD146-PDGFRβ cells were cultured as monolayers or micromass pellets (chondrogenic) for four weeks in differentiation induction or control media. (A) Osteogenic-differentiated cells express alkaline phosphatase (left panel) and osteoblastic lineage-specific genes. (B) Adipogenic-differentiated cells are visualized as oil red O-stained fat droplets (left panel) and express adipocyte-specific genes. (C) Myogenic-differentiated cells express smooth muscle proteins, α-SMA and smooth muscle-specific myosin heavy chain (MHC), and calponin and caldesmon mRNA. (D) Chondrogenic-differentiated cells in a micromass pellet section showing Alcian blue-stained cartilage matrix (left panel) and mRNA for chondrocyte lineage-specific genes. (E) CD146 and PDGFRβ co-localize on perivascular cells of blood vessels (white arrows) in the functional (shown) and basal layers. Co-localization of the two surface markers is shown in the x/z and y/z planes of the merged images. Source: From Ref. 40.

factor and marker of pluripotent human embryonic stem cells (55) and, more recently, of adult stem cells (56), was expressed in some human endometrial samples (57). More OCT-4+ cells were observed during the proliferative stage. However, this study did not quantit...
Figure 5  Schematic showing the possible location of candidate endometrial stem/progenitor cells in human and mouse endometrium. (A) In human endometrium, it is hypothesized that epithelial stem/progenitors are located in the base of the glands in the basalis. Recent data indicates that MSC-like cells are located near blood vessels possibly in both the basalis and/or the functionalis. (B) In mouse endometrium, candidate epithelial and stromal stem/progenitor cells (label-retaining cells, LRC), which rapidly proliferate during estrogen-stimulated endometrial growth, are located in the luminal epithelium and mainly near blood vessels at the endometrial-myometrium junction, respectively. Source: From Ref. 54.

determine the identity or location of the OCT-4+ cells. Pou class 5 transcription factor 1 (pou5f1) was co-localized in 0.19% of stromal LRC in the lower region of the murine endometrial stroma, suggesting the highly undifferentiated status of this specific subset of LRC (54). Nested PCR confirmed the presence of Pou5f1 mRNA in mouse uterus during the prepupal period (day 21) and in adulthood (day 50) (54).

Musashi-1, an RNA-binding protein in neural stem cells and an epithelial progenitor cell marker that regulates stem cell self-renewal signaling pathways (58), was recently localized to single epithelial and stromal cells as well as small clusters of stromal cells in human endometrium (59). Musashi-1-expressing cells were greater in number in the basalis compared with the functionalis in the proliferative stage of the menstrual cycle, suggesting their possible stem/progenitor cell function. Notch-1 and telomerase, key downstream targets of Musashi-1, co-localized with Musashi-1+ endometrial cells. Telomerase activity has been identified in adult stem cells in other tissues (60). Interestingly, the stromal Musashi-1+ cells were not found in a perivascular location (42), although some were found in a periglandular region, similar to some stromal LRC in mouse endometrium (33). It is now important to determine whether Musashi-1 is expressed in endometrial stromal CFU and in CD146+PDGF-RB+ cells, although the latter would appear unlikely given their different localizations.

A recent flow cytometric analysis identified cells with a hematopoietic stem cell phenotype (CD34+CD45+) in human endometrial cell suspensions that co-expressed CD7 and CD86 and appear to be lymphoid progenitors (61). Whether these cells function as hematopoietic stem cells and generate endometrial leukocytes in the endometrium or contribute to the SP is unknown.

A stem cell marker of hematopoietic stem cells is the proto-oncogene c-KIT (CD117), which encodes a 145-kDa transmembrane tyrosine kinase receptor specific for its ligand stem cell factor (62). While c-KIT was co-localized to 0.32% of stromal LRC in the lower region of murine endometrium (34), it was not detected in endometrial stromal LRC in another study (35). Neither human endometrial epithelial nor stromal CFU responded to stem cell factor in CFU assays, suggesting that c-KIT may not be important in the function of human endometrial stem/progenitor cell function (24,25).
Indirect Evidence for Endometrial Stem/Progenitor Cells

Monoclonality of Endometrial Glands

Evidence indicates that endometrial glands are monoclonal in origin, suggesting that they arise from a single progenitor or stem cell. In almost half of histologically normal proliferative endometrial samples, rare glands have been observed that fail to express phosphatase and tensin homolog (PTEN) protein (PTEN-null glands) because of a mutation of and/or deletion in the PTEN gene (63). These PTEN-mutant glandular clones persist in the basalis region between menstrual cycles to regenerate their respective glands in the functional layer in subsequent cycles. PTEN-null glands are increased in the endometrium of women in conditions arising from unopposed estrogen, particularly endometrial hyperplasia, a monoclonal epithelial proliferative disorder (64,65). In a separate study, monoclonality was detected in carefully dissected individual endometrial glands using a polymerase chain reaction (PCR)-based assay for nonrandom X chromosome inactivation of the androgen receptor gene (66). Furthermore, adjacent glands up to 1 mm apart shared clonality, indicating that well-circumscribed regions of endometrium were derived from the same precursor cell, suggesting that several glands share the same stem cell. This raises questions on the precise locality of candidate human epithelial stem/progenitor cells.

Gland Methylation Patterns

Another retrospective approach indicating adult stem/progenitor cell activity in endometrium analyzed methylation patterns in endometrial glands (67). Epigenetic changes to DNA sequences arising during cell division encode a cellular history in individual glands, which reflect the methylation patterns arising in resident stem/progenitor cells, since these are inherited in subsequent cell divisions and retained. In contrast, those arising in more mature progeny are lost during shedding. Recently, methylation patterns observed in individual glands from cycling and atrophic human endometrium were subjected to mathematical modeling, which supported the concept that an individual gland contains a stem cell niche with an unknown number of long-lived stem cells rather than a single stem cell (67). Further evidence of gland diversity in aging endometrial glands indicated that a reservoir of stem cells remained in atrophic endometrium, supporting the data from clonogenicity studies of human endometrium (25).

Source of Endometrial Stem/Progenitor Cells

Remnant Fetal Stem Cells

The embryonic female reproductive tract has its origins in the intermediate mesoderm, which begins to form soon after gastrulation. As this embryonic tissue proliferates, it is thought that some cells undergo mesenchymal to epithelial transition to give rise to the coelomic epithelium that later invaginates to form the paramesonephric or müllerian ducts (68). These ducts comprise surface epithelium and underlying urogenital ridge mesenchyme. During fetal life, the glands commence developing as the undifferentiated uterine surface epithelium invaginates into the underlying mesenchyme, and the inner myometrium commences to form as smooth muscle cells differentiate from the mesenchyme (10).

A small number of fetal epithelial and MSC are thought to remain in the adult endometrium and contribute to tissue replacement during its cyclic regeneration (69). Whether there is an ultimate uterine stem cell that has the capacity to replace all endometrial and myometrial cells, including epithelial, stromal, vascular, and smooth muscles, is whether there are separate epithelial and MSC is not currently known. The different phenotypes, growth factor dependence, and frequency of clonogenic endometrial epithelial and stromal cells suggest that there are at least two endometrial progenitor cells. However, this does not exclude the possibility of an unidentified, more primitive precursor in human endometrium.

Circulating Stem Cells from the Bone Marrow

There is increasing evidence that bone marrow-derived cells may also be a potential source of cells for endometrial regeneration (70-72). Significant chimerism ranging from 0.2% to 52% was detected in the endometrial glands and stroma of four women who received single-antigenes
histocompatibility locus antigen (HLA)–mismatched bone marrow transplants, suggesting that bone marrow stem cells contributed to endometrial regeneration in a setting of cellular turnover and inflammatory stimuli (69). It is not known if the source of the donor bone marrow cells contributing to chimeric endometrial tissue is hematopoietic or MSC. Further evidence for bone marrow stem cell contribution to endometrial repair comes from gender-mismatch bone marrow transplant studies in mice, where less than 0.01% of cytokeratin-positive endometrial epithelial cells and less than 0.1% of stromal cells contained a Y chromosome (72). Bone marrow cell contribution to endometrial repair is very modest, and engraftment of the endometrium seems more likely during repair after injury. Bone marrow cell contribution to endometrial regeneration may also have a role in the extensive endometrial epithelial growth and regeneration occurring during pregnancy and after parturition. Recently, circulating CD45+ bone marrow cells were shown to contribute 82% of mouse uterine epithelium during pregnancy in a novel double reporter CD45Cre-Z/EG transgenic mouse used to track the fate of CD45+ green fluorescent protein (GFP) cells in female mice (71). These preliminary results need to be interpreted with caution as data was obtained only from a single pregnant reporter mouse. However, small but increasing numbers of GFP+ endometrial epithelial cells were also observed in the luminal epithelium as the mice aged, ranging from 0% in 1- and 5-week-old to 0.5% in 12-week-old and 6% in 20-week-old mice (71). Although there were insufficient animals per group for statistical analysis, these data suggest increasing contribution of bone marrow-derived cells to the endometrial epithelium over time. Clonal expansion of transplant donor cells in the endometrium was apparent as GFP+ cells were often found in clusters Endometrial epithelial and stromal LRC did not express CD45 (33), but expression of this hematopoietic marker may be lost if bone marrow cells incorporate and transdifferentiate into endometrial epithelium. The role of estrogen and progesterone in recruiting bone marrow cells has not been examined, although progesterone may have a role during pregnancy.

ENDOMETRIAL STEM/PROGENITOR CELLS: CLINICAL PERSPECTIVE
Since adult stem cells regulate tissue homeostasis, it is expected that abnormal functioning of endometrial stem/progenitor cells and/or their surrounding niche cells may also be involved in the initiation and progression of gynecological diseases associated with abnormal endometrial proliferation, such as endometriosis, adenomyosis, endometrial hyperplasia, and endometrial cancer (17). Furthermore, epithelial and stromal CFU are present in non-cycling and perimenopausal endometrium (25) and may be responsible for regenerating endometrium in women given estrogen replacement therapy (17).

Cancer Stem Cells in Endometrial Cancer
The cellular composition of any cancer is quite heterogeneous. Individual tumor cells vary in their ability to initiate tumors, their expression of markers and level of differentiation, their ability to generate tumor cells, and their life span. Like their normal tissue counterparts, there is a cellular hierarchy in tumors with the rare stem cell or cancer stem cell (CSC) at the apex (79). CSC have been extensively characterized in leukemias and are currently being characterized in many solid human tumors including breast, glioblastoma, colon, pancreas, prostate, and ovary (74). Controversy exists on the origin of the CSC: is it transformation of a normal adult stem cell, or do more mature cells gain mutations that enable them to acquire self-renewal properties of adult stem cells? A CSC is defined as a self-renewing cell within a tumor that has the capacity to regenerate the phenotypic diversity of the original tumor (74). Thus, CSC are able to initiate, maintain, and propagate tumors in vivo producing heterogeneous tumor cell progeny, initiate clones in vitro, undergo self-renewal cell divisions, and have high proliferation potential. In cancers where CSC have been identified, they comprise less than 1% of the total cell population.

Endometrial cancer is characterized by abnormal endometrial epithelial cell proliferation. It affects around 6430 women each year in the United Kingdom, resulting in approximately 1630 deaths (75). Endometrial cancer is the most common gynecological malignancy in the western world. There are two types of endometrial adenocarcinoma. Type I generally effects pre- and perimenopausal women, is estrogen dependent, and is associated with mutations in PTEN, K-RAS, and β-catenin genes or MSI (microsatellite instability) (76). Type II normally
affects postmenopausal women, is estrogen independent, and is associated with mutations in p53 and HER-2/neu (76).

Evidence for endometrial CSC has recently been obtained from studies in human endometrial cancer cell lines and isolated primary endometrial cancer cells (77,78). The endometrial cancer cell line, AN3CA, possessed a small population of SP cells (0.02%) (77). Further investigation revealed that AN3CA SP cells were relatively quiescent as they were mainly in the G1 stage of the cell cycle and had low proliferative activity during the initial stages of cell culture. The AN3CA SP cells displayed adult stem cell functional properties of self-renewal and differentiation in vitro as they maintained similar proportions of SP cells after many passages in culture. The AN3CA SP cells were also relatively resistant to the chemotherapeutic agent paclitaxel compared with the MP cells (77). It remains to be seen if these properties are present in tumor cells isolated from primary human endometrial carcinomas. Tumor-initiating cells were also demonstrated in the SP cells but not MP cells after both cell fractions were injected subcutaneously into male NOD/SCID mice with estrogen implants (77). All of the SP injections resulted in tumors, whereas the MP injections failed to produce tumors (77).

CSC as tumor-initiating cells have also been demonstrated in unsorted cells isolated from primary human endometrial carcinomas, which have been transplanted into NOD/SCID mice either subcutaneously (77) or under the kidney capsule (78) and produced tumors in both locations. When transplanted in limiting dilution (10³–10⁶ cells), not all dilutions resulted in tumors, suggesting that not all cells within the tumor have the same capacity to initiate tumor growth (78). Similarly, not every cell within the tumor was able to produce a clone in vitro in cell cloning studies (78), evidence supporting the concept of the cellular heterogeneity of primary endometrial carcinomas. An alternative explanation is that some tumor cells lack the ability to adapt to the new environment in vitro and in vivo, and hence ability to grow and proliferate.

In vivo self-renewal of endometrial CSC was demonstrated by the formation of new secondary tumors after grafting cells isolated from the original tumor transplants (77). Fewer tumor cells were required to initiate tumors on successive serial transplantation of secondary and tertiary tumors, indicating that increasing numbers of CSC with self-renewal capacity were produced. Self-renewal of endometrial CSC has also been demonstrated in vitro by serial cloning of initial individual CFU recultured at clonal seeding densities (<10 cells/cm²) (78). Secondary, tertiary, and quaternary clones were produced. The fact that only some cells re-cloned indicates that only a few cells within a clone have self-renewal capacity.

The transplanted endometrial cancer cells produced tumors with similar histology and phenotype as the parent tumor (Fig. 6) (77,78), indicating the ability of the tumor-initiating cells to differentiate into the heterogeneous mix of cells comprising the original tumor. Differentiation was demonstrated by investigating differentiation markers by immunohistochemistry. The distribution of EpCAM⁺ epithelial cells was similar in parent tumor and transplants, and the pattern of cells expressing ERα and PR was similar to that observed in the parent tumor (78). While rare cells isolated from primary human endometrial cancers have CSC properties of tumorigenicity, self-renewal, differentiation, and clonogenicity, markers of these endometrial CSC have not yet been discovered. Identification of such markers will allow the prospective isolation of endometrial CSC and investigation into their role in the development and progression of endometrial carcinoma.

Endometriosis
Endometriosis is characterized by the growth of ectopic endometrial tissue on pelvic organs and the peritoneum (79). It is thought that retrograde menstruation, which occurs in most menstruating women, deposits menstrual debris into the peritoneal cavity. However, it is not known why only 6% to 10% of women develop endometriosis and its associated symptoms of inflammation, pain, and infertility. It has been postulated that in women who develop endometriosis, endometrial stem/progenitor cells are inappropriately shed during menstruation and reach the peritoneal cavity where they adhere and establish endometriotic implants (17,26,80). This assertion is supported by the demonstration of clonogenic cells in a long-term culture derived from a sample of endometriotic tissue (81) and monoclonality of some
endometriotic lesions (82). Bone marrow stem cells may contribute to the progression of endometriosis lesion development as demonstrated recently in a mouse model (72). Some forms of endometriosis may arise from remnant fetal mullerian cells, which may behave like stem cells to establish ectopic growth of endometrial tissue. Clearly, the role of endometrial stem/progenitor cells or bone marrow stem cells in the development of endometriosis will require an extensive research effort.

Adenomyosis

Adenomyosis, a condition affecting 1% of women, results from extensive myometrial invasion by the basal endometrium. It is associated with smooth muscle hyperplasia and is also considered to arise from fetal mullerian cells (83).

It is possible that endometrial stem/progenitor cells or their niche cells demonstrate abnormal behavior in adenomyosis, or these putative stem cells have an abnormally orientated niche such that their differentiating progeny are directed toward the myometrium rather than...
functional, producing pockets of endometrial tissue deep within the myometrium. Alterations in the putative endometrial stem cell niche, particularly in the niche cells regulating stem cell fate decisions may result in excessive smooth muscle differentiation of putative endometrial stem/progenitor cells producing the observed myometrial hyperplasia. Much research is required to establish a role for endometrial progenitors or myometrial SP cells in the pathogenesis of adenomyosis.

Tissue Engineering Applications
There is great interest in the use of both embryonic and adult stem cells in tissue engineering applications for restoring function to aging or diseased tissues and organs. Medical advances have ensured increasing longevity, and the aging population has many tissues in need of repair (84). The failure of artificial implants to last longer than 10 to 15 years and the problems associated with nondegradable synthetic materials make cell-based therapies for tissue replacement attractive (85). There is now a focus on using a combination of temporary biological scaffold materials to provide initial support and stem cells to promote appropriate tissue genesis and regeneration of functional tissue (85). This is particularly important for the provision of supportive tissues and could be adapted for tissue engineering support of the female reproductive tract. Pelvic floor prolapse is a major problem resulting in 10% of women requiring surgery, with approximately 30% of these requiring repeat surgery (86). The use of artificial and biological scaffolds for pelvic floor prolapse surgery has improved outcomes to a limited degree. Thus, the use of tissue constructs comprising scaffolds and autologous endometrial mesenchymal stem/progenitor cells may provide a possible solution for treatment of pelvic floor prolapse in future (26).

CONCLUSIONS AND FUTURE DIRECTIONS FOR HUMAN ENDOOMETRIAL STEM/PROGENITOR CELL RESEARCH
There is now sufficient evidence to conclude that rare populations of adult epithelial and mesenchymal stem/progenitor cells exist in normal human and mouse endometrium and rare CSC are present in endometrial adenocarcinoma. While some evidence has been obtained from in vivo studies in mice, identifying candidate stem/progenitor cells as LRC, all evidence collected to date for human endometrium has been from in vitro studies. It is most important that the capacity of these rare cells to reconstitute endometrial tissue in vivo using xenotransplantation approaches is undertaken. Such a model has already been published and could be adapted to examine putative endometrial stem/progenitor populations (49). Whether there is a single more primitive endometrial stem cell that produces all cell types in the uterus is yet unknown. Whether the bone marrow is a source of endometrial stem/progenitor cells as a major or minor contributor needs to be determined under physiological conditions. There is also a pressing need to identify definitive markers for endometrial epithelial stem/progenitor cells and find markers that further purify the CD146/PDGFR-β/ endometrial MSC-like cells. Further characterization of the endometrial stem cell niches and the signaling pathways involved in the regulation of the resident stem/progenitor cells is also required. Investigation into the possible roles of developmental pathways involving bone-morphogenetic protein, Hedgehog, Notch, and Wnt signaling in endometrial stem/progenitor cell self-renewal and cell fate differentiation decisions, would be a valuable starting point as these molecules or pathways have already been detected in endometrium or have important roles during endometrial development or deciduization when stromal cells undergo terminal differentiation. More extensive studies examining how estrogen, progesterone, and the growth factors EGF, TGFβ, PDGF, and bFGF interact with endometrial stem/progenitor cells and their niche cells would be useful. How estrogen and progesterone interact with endometrial stem/progenitor cells or their neighboring niche cells needs to be explored.

Such additional knowledge will assist the investigation into the role of endometrial stem/progenitor cells in gynecological disorders associated with abnormal endometrial proliferation and will not only increase our understanding of the pathophysiology of endometriosis, adenomyosis, endometrial hyperplasia, and endometrial cancer but it also has the potential to change the way these hormone-dependent diseases will be treated in future.
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